IN-VITRO MODELLING OF LYMPHOMA - POTENTIAL FOR EXPLORING EXPERIMENTAL THERAPIES

by

Jonathan Shamash

MD

University of Edinburgh

1996

ABSTRACT

The development of new therapies for the treatment of lymphoma has been hampered by the lack of reproducible and faithful *in vitro* models. This has been a particular problem with the relatively slow growing types of which two broad groups are considered here - namely the low grade B cell non-Hodgkin's lymphomas and Hodgkin's disease.

This thesis describes the development of a culture system using a stromal cell layer, anti-CD40 and two cytokines - human interleukin 3 (IL3) and human interleukin 10 (IL10) which has resulted in the *in vitro* expansion of low grade B cell malignancies with retention of some of the morphological and immunophenotypical features of the parent tumour and in addition a growth rate similar to that found *in vivo.* This system was able to support the growth of Hodgkin's lymphoma - both lymphocyte predominant and non lymphocyte predominant subtypes. Molecular analyses of these cells suggested a B cell origin in most cases and cell sorting using flow cytometry for CD30 positive (CD30+ve) cells revealed the presence of Reed Sternberg cells (RS cells) or lymphocytic and/or histiocytic (L + H) cells in the CD30+ve cell sorts.

A variant of this culture system using human interleukin 4 (IL4) which has already been shown to support the growth of follicle centre lymphoma was found to upregulate CD80 (B7-1), creating the opportunity to assess the potential for an immunotherapy based approach, as the expression of this accessory antigen is thought by some to be crucial to the development of an adequate immune response. This was tested in a mixed lymphocyte reaction - firstly using allogeneic lymphocytes and then with autologous

ones against lymphoma cells which prior to culture did not express CD80, and then with cultured cells which did. Cells cultured using IL3 and IL10 also expressed CD80 although to a lesser degree and the potential for these cells to elicit mixed lymphocyte responses was also assessed.

The IL3/IL10 anti-CD40 stromal cell system was then used to assess conventional chemotherapeutic drugs and the findings were compared to those in a transformed follicle centre lymphoma cell line.

The knowledge that the anti-apoptotic protein *Bcl-2* prevented intracellular repartitioning of calcium lead to the assessment of calcium channel blocking drugs acting at two sites (1) the cell membrane (nimodipine) and (2) the endoplasmic reticulum (dantrolene and azumolene). These were initially tested in various lymphoma cell lines with differing levels of *bcl-2* expression and then in primary lymphoma culture. The effect of these drugs alone and in combination with cytotoxic agents was compared and the *in vitro* cytotoxic activity of dantrolene and azumolene described.

The contribution of such a system for primary lymphoma culture to the assessment of therapy - both pharmacological and immunotherapeutic is considered, as is the potential for further unravelling the mysteries of Hodgkin's disease.

CONTENTS

ACKNOWLEDGEMENTS

The work described in this thesis was carried out in the main at the Imperial Cancer Research Fund laboratories of the Medical Oncology Unit at St Bartholomew's Hospital under the supervision of Professor Andrew Lister and Professor Bryan Young between June 1993 and December 1995.

Professor Lister was kind enough to review the work as it progressed and I am grateful for his encouragement and advice particularly when experiments were not going well. Professor Young was very helpful in the interpretation of the molecular findings in the Hodgkin's lymphoma cultures.

Various sections of this thesis depended on the advice of others, in particular, I should like to thank Professor Peter Beverley for his advice on primary lymphoma cultures and the CD80 based experiments, Dr Diana Wallace for telling me how to set up the mixed lymphocyte reactions and interpret the results. Dr Andrew Norton kindly reviewed numerous cytocentrifugation specimens which helped to confirm that the morphology of the various tumours was being conserved. Derek Davies in the ICRF FACS laboratory, Lincoln's Inn Fields provided ideas and assistance in interpreting FACS data and carrying out cell sorts, Ashiq Salam in Medical Oncology helped to prepare the lymphoma tissue for culture and was responsible for maintaining the cell line experiments, some of which required considerable time and attention.

The work described was carried out in the main by myself, with the exception of the PCR analysis and sequencing in Chapter 2 which was performed by Dr Vassiliki Pappa and the P-Glycoprotein experiments in

Chapter 5 carried out by Amanda Williams under the supervision of Simon Joel at the Barry Reid Oncology Laboratory at St Bartholomew's Hospital, he also assisted me in the statistical analyses.

Finally, I should like to thank the Department of Radiotherapy at St Bartholomew's Hospital for irradiation of the stromal cells - often at short notice.

20/3/96

"..... the eye shall not be sated from seeing, nor shall the ear be filled from hearing. That which has been is the same at that which will be, and that which has been done is the same as that which will be done, and there is nothing new under the sun. There is a thing of which (someone) will say, 'see this, it is new.' It has already been for ages which were before us."

Ecclesiastes (Kohelet) 1. 8-10.

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CHAPTER 1

LYMPHOMA - HISTORICAL PERSPECTIVES, CLASSIFICATION AND TREATMENT OUTCOME - WITH SPECIAL EMPHASIS ON LOW GRADE B CELL AND HODGKIN'S LYMPHOMA

1. LYMPHOMA - HISTORICAL PERSPECTIVES, CLASSIFICATION AND TREATMENT OUTCOME - WITH SPECIAL EMPHASIS ON LOW GRADE B CELL AND HODGKIN'S LYMPHOMA

1.1 Background and classification of Lymphoma

Lymphoma - malignant disease arising from lymphoid tissue has confounded pathologists and clinicians for the past 150 years, with attempts being made to link the features seen at microscopy with the overall clinical behaviour of disease in an individual.

In 1832 Thomas Hodgkin gave a clear description[1] of a primary lymphoid disorder involving the spleen and lymph nodes. Not all cases were of the lymphoma now eponomously linked to him but some clearly were. In 1845 Bennet described a disorder which linked peripheral blood leukocytosis to enlargement of lymph node, spleen and liver. It was not until 1864 that Virchow used the term lymphosarcoma to encompass Hodgkin's and non-Hodgkin's lymphomas linking these conditions[1], and in 1871 Billroth used the name 'malignant lymphoma' as a loose term to describe these diseases. Sternberg in 1898 having described the presence of tuberculosis in the context of large multinucleated cells which now carry his name felt that Hodgkin's disease was an atypical form of tuberculosis. By 1901, Sir Frederick Andrewes of St Bartholomew's Hospital, London dismissed this and felt it was a discrete entity, Dorothy Reed went further to suggest that it was able to metastasise - pushing it towards classification within malignant disease and in 1931 Warthin described it as being a

neoplasm[1]. Gordon[2] in 1932 innoculated rabbits intra cerebrally with Hodgkin tissue producing encephalitis suggesting a transmissible agent. Although similar effects could be produced by benign lymphoid tissue infected with trypanosomes, but not normal benign tissue, the failure to transfer the encephalitis from one rabbit to another detracted from the finding.

Maximow[1] felt that lymphoid tumours arose from pluripotential stem cells which he termed reticulum cells, and the tumours arising from them - reticuloses. In the United States Brill, Baehr and Rosenthal described a clinical entity in 1925[3] - clearly follicle centre lymphoma and Symmers[4] described follicular adenopathy and splenomegaly in 1927, this acquired the eponym Brill Symmer's disease, although some of the cases Symmers described included other causes of lymph node enlargement. The disease was eventually fatal with an aggressive form arising after several years of relatively asymptomatic activity.

The balance between those who believed that lymphomas were just results of the same primary process which could pass from one form to another, or descrete entities, shifted back towards the latter with Robb-Smith in 1938 in his classification of lymphoid disorders identifying follicular reticulosis and Hodgkin's disease as well as a variant which would become known as Hodgkin's Paragranuloma and eventually lymphocyte predominant Hodgkin's lymphoma.

Paragranuloma and Granuloma were names applied by Jackson and Parker to types of Hodgkin's lymphoma and their conclusion that cases of paragranuloma which failed to evolve into granuloma may be considered to be a separate disease can be seen as an early attempt to separate Lymphocyte Predominant Hodgkin's Lymphoma (LP-HL) from the other types.

Attempts to separate the various lymphomas on clinical grounds were further enhanced with Sir Ronald Bodley Scott suggesting that there were 'several distinct morbid processes each with a different clinical course'[1]. Rappaport et al suggested considerable variability within the clinical group of follicle centre lymphoma and also distinguished Hodgkin's disease from other lymphomas. In 1966 his classification separated lymphomas into diffuse or nodular types with a specific Hodgkin's type. Lukes and Butler subdivided Hodgkin's lymphoma into 6 types with the Rye classification in 1965 - reducing this to 4 main types. The staging system which was adopted for Hodgkin's lymphoma was devised at Ann Arbor in 1971, with the Cotswold's modification[5] introduced in 1989 to further designate bulk disease, and assist in the classification of complete responses, bringing in the term CR(U) - uncertain complete remission - when clinical remission existed in the presence of uncertain radiological features. The most recent attempt to classify lymphoma[6] - the Revised European, American Lymphoma Classification (REAL) has essentially left the classification of Hodgkin's lymphoma unchanged - maintaining lymphocyte predominant Hodgkin's lymphoma within the group

and adding a further provisional entity Lymphocyte Rich - classical Hodgkin's.

Agreement as to how to classify the non-Hodgkin's lymphomas has been less forthcoming. In 1942 Gall and Mallory[7] classified NHl into 3 main groups: 1) Follicular, Lymphosarcoma Lymphocytic (CLL); 2) Lymphosarcoma Lymphoblastic; 3) Reticulum Cell Sarcoma (plasma cell derived). In 1966 Rappaport introduced his morphology based classification and in 1968 - the concept of deriving malignancies from B or T cell precursors was introduced and the germinal centre cell origin of follicle centre lymphoma was exemplified by Lennert in 1971. Lukes and Collins introduced a classification based on B or T cell origin and on morphology in 1974. Lennert et al more influenced by clinical entities described subtypes again based on B or T cell origin. The division into low and high grade tumours was proposed by Bennet and in 1974 a group of pathologists at Kiel maintained this division placing tumours derived from T or B cell sources in a low or high grade category[1]. The number of competing classifications lead to an attempt to work out a conversion system between them. This was known as the Working Formulation - clinical division being into low, intermediate or high grade - with each grade having prognostic significance with different subtypes listed within each group. While 'clinician friendly' - and useful for therapy planning, this formulation which was never intended as a classification as such failed to take account of aetiology and derivation of the various tumours and the recent description of new clinical entities e.g. Mantle Cell Lymphoma, Mucosa Associated Lymphoid Tissue

Tumours, CD30+ve Anaplastic Large Cell Lymphoma and Primary Mediastinal Large B Cell Lymphoma. The failure of any of the current systems to have gained widespread acceptance and the realisation that not all the subtypes classified under the various schemes can be reproducibly classified as such by different pathologists encouraged the establishment of what has been termed the REAL classification[6]. This lists clinical entities with common morphology, behaviour and immunological and in some cases genetic similarities. It leaves some areas unclassified, inserts provisional entities and makes the grading of tumours optional. The discovery that some 'low grade' tumours like mantle cell lymphoma[8] (centrocytic-Kiel) have very poor prognoses much worse than follicle centre lymphomas has suggested that each tumour be graded separately e.g. follicle cell lymphoma - grade 1-3 - rather than lumped in a group which presumes a degree of clinical aggressiveness. Whether sufficient agreement can be reached to allow the adoption of this scheme remains to be seen, but its formal recognition of lymphoma entities outside the older classifications is certainly an advantage and its acceptance by pathologists and clinicians may well allow easier comparisons of clinical outcome to be made.

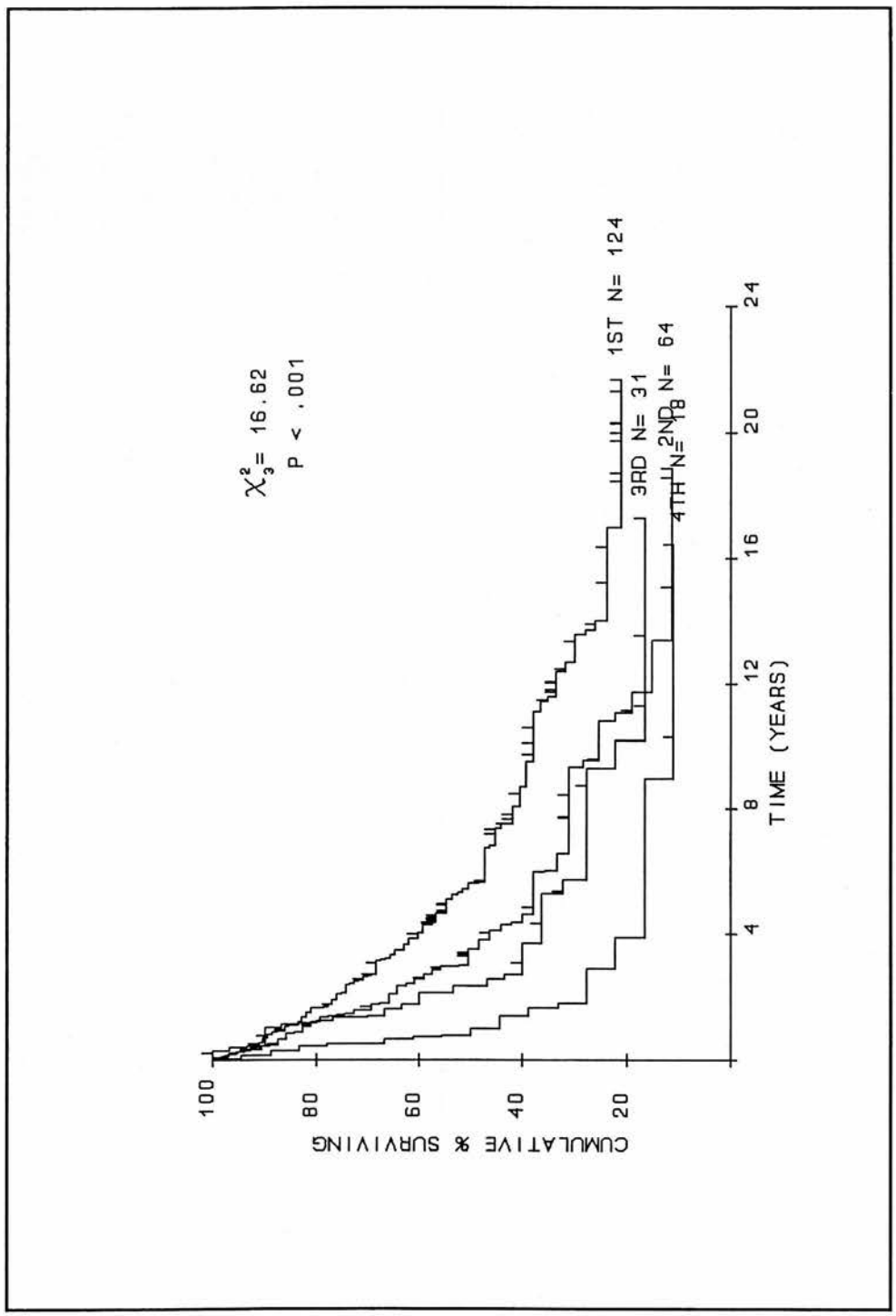
1.2 Clinical outcome of low grade B cell lymphomas and Hodgkin's Disease

The low grade B cell lymphomas include follicle centre lymphomas, lymphoplasmacytoid lymphomas and small cell lymphocytic lymphomas (REAL classification). Follicle centre lymphoma is the commonest and the tendency of all is to relapse

and remit. 40 years ago median survival was expected to be about 5 years[9]. Transformation to high grade histology was known to supervene. Median survival in the last 5-10 years has now increased to 9-10 years[9]. Retreatment following initial relapse normally results in further remission each remission lasting 2-3 years unless transformation should occur, when the median survival falls to 8-10 months. Most patients present with stage III/IV disease (75%), those presenting with stage I or II have a better outcome. In these patients involved field radiation and multiagent chemotherapy (cyclophosphamide, vincristine and prednisolone) has resulted in an 83% relapse free survival at 5 years[10].

For advanced disease there appears to be little advantage of multidrug treatment over single agent chlorambucil[11]. The addition of an anthracycline in initial treatment appears to have doubtful value except for those within an excess number of blast cells[9]. Interestingly, failure to respond to a treatment on the first occasion does not preclude response on a subsequent occasion[10]. After 1st relapse median survival is in the region of 5 years[10].

Survival from recurrences of patients with follicular non-Hodgkin's lymphoma



The poor long term outlook of these conditions has encouraged the search for alternative approaches. The purine analogues fludarabine and cladribine - both deoxyadenosine analogues are taken up by lymphoid cells where they induce apoptosis[12]. In previously treated patients (3 regimens on average) treatment with fludarabine has yielded a response rate of 48%[13], Interferon α has a 40% response rate in untreated patients[9] and maintenance treatment with interferon to people in remission induced by chemotherapy prolongs disease free survival[9]. As this group of diseases is chemosensitive, high dose intensification with cyclophosphamide and total body irradiation for patients in 2nd or subsequent remission has been offered[14] with purged autologous bone marrow support. This treatment has resulted in a 5% toxic death rate and 5% late death rate from secondary myelodysplasia/acute myeloid leukaemia[15]. Although prolonged remissions occur whether this treatment can be considered curative remains an open question.

New approaches using targeted monoclonal antibodies[16] have been encouraging and the activity of interferon has enhanced the cause of immunotherapy. The importance of *bcl-2* in the suppression[17] of apoptosis that occurs in these tumours has encouraged the development of antisense oligonucleotides targetted to the translocation breakpoints that result in its upregulation.

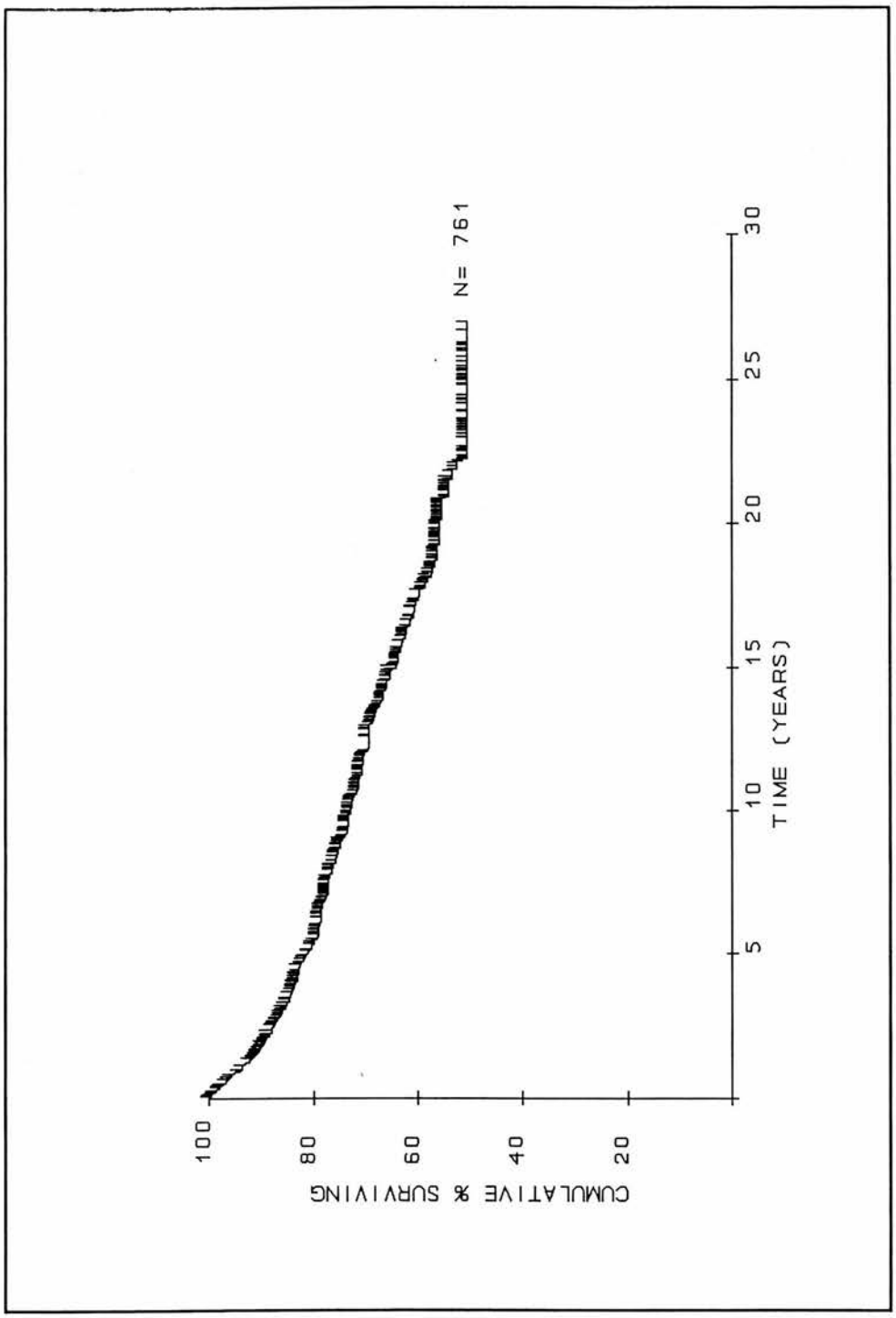
Improvements in *in-vitro* modelling would aid the assessment of new treatments and the primary culture of low grade lymphoma

described has allowed a potential immunotherapy based strategy and a pharmacological approach to be examined in this thesis.

1.3 Hodgkin's Lymphoma

The clinical outcome in HL remains better than low grade NHL. Median survival at a single centre[18] has been reported as 18 years. The progression of the disease from one nodal group to another has lent itself to be use of irradiation to encompass the likely sites of nodal disease. The median survival following relapse is the same regardless of the modality of initial therapy. The lymphocyte predominant group appears to have a better prognosis than the rest[19] - accounting for only 7% of the total cases, it presents at an earlier stage with a 12 year survival of 82%, however 12% of patients developed secondary malignancies over 13 years which is high, 1/3 being high grade undifferentiated B cell lymphomas. Chemosensitivity to first line treatment governs the likelihood of response to subsequent conventional treatment[18]. The original MOPP like treatments achieve complete response rates of 70% in advanced disease; 70% of these will be durable. For those who relapse after initial complete response 81% will respond again.

Overall survival of Hodgkin's Lymphoma at St Bartholomew's Hospital



Addition of an anthracycline to the primary treatment has improved the survival with MOPP-ABVD or ABVD alone being superior to MOPP - 5 year survival being 75, 73 and 66% respectively[20]. Failure to remit carries a poor prognosis with a median survival of 1.4 years[18].

The role of high dose treatment in relapsing patients is unclear. The timing of its use has depended on the enthusiasm of the treating clinician. Poor prognosis suggested by those whose 1st remission is less than 1 year - would appear to be a reasonable indication[18], however failure to respond to treatment does not imply no benefit from high dose chemotherapy as some of these patients will derive prolonged disease free survival and thus some feel it should be given to those refractory to conventional treatment[21], median progression free survival in those failing to attain complete remission being 42% at 3.6 years[22].

The tendency to relapse with chemosensitive disease is reminiscent of the situation with low grade NHL. However, the time scales are different with median life expectancy with HL being about twice that of low grade NHL. Both tumours have generally low growth rates - a situation which is difficult to mimic with cell lines. In HL most of the cells in the affected node are benign[23] with perhaps only 1-2% being malignant cells a situation which is unusual in cancer.

New approaches to treatment have included monoclonal antibodies targeted at the Reed Sternberg-Hodgkin's (RS-H) cells

using anti-CD30 and anti-CD25[24] conjugated with saporin. Excellent responses have been seen but as the antibodies to date have been murine repeated exposure leads neutralising antibodies forming and subsequent relapse. The role of antibody treatment in minimal residual disease remains to be investigated.

Attempts to understand the biology of the RS-H cells and the relationship between LP-HL and the rest are hampered by poor *in-vitro* models. The stromal cell system described has allowed further analysis of primary cultured cells to be made and will hopefully lead to an enhanced understanding of the disease.

CHAPTER 2

IN VITRO CONTROL OF LYMPHOCYTE PROLIFERATION AND DIFFERENTIATION - A BASIS FOR DESIGNING THE STROMAL CELL SYSTEM

2. IN VITRO CONTROL OF LYMPHOCYTE PROLIFERATION AND DIFFERENTIATION - A BASIS FOR DESIGNING THE STROMAL CELL SYSTEM

2.1 Introduction

2.2 CD40

2.3 Cytokines and B cell proliferation and differentiation - IL3, IL4 and IL10

2.4 Rationale for cytokine combinations in the stromal cell system

2. IN VITRO CONTROL OF LYMPHOCYTE PROLIFERATION AND DIFFERENTIATION - A BASIS FOR DESIGNING THE STROMAL CELL SYSTEM

2.1 Introduction

This chapter reviews the various triggers and cytokines required to induce B cell proliferation and differentiation and concludes with the rationale for the stromal cell system subsequently investigated.

2.2 CD40

The CD40 antigen has an important role in B cell activation, proliferation and class switching[25], it is a 47-50KD glycoprotein, a member of the tumour necrosis factor (TNF) ligand superfamily[26] and exists in a soluble form. As well as its presence on B cells it has also been detected on epithelial cells, basophils, and follicular dendritic cells[27], it is upregulated after activation and down regulated as B cells undergo terminal differentiation. CD40 may be upregulated by IL4, IgM, and anti-CD40[28]. Ligation leads to proliferation of anti IgM crosslinked or IL4 stimulated B cells.

Its natural ligand (CD40L, gp39) is found on T cells[29], failure of this to bind appropriately leads to the hyper IgM syndrome and the lack of normal B cell proliferation *in vitro*. The CD40 antigen in these patients is normal[29].

The crosslinking of CD40 - either using antibody directed against it or the CD40L leads to the suppression of apoptosis[30]. This effect is partly mediated by *bcl-2*, but as constitutive *bcl-2* production does not rescue sIg mediated apoptosis another mechanism must be required. CD40 signalling appears to required tyrosine phosphorylation[25].

CD40 binding is important in modulating the response of B cells to cytokines. Benign tonsillar B lymphocytes will undergo long term proliferation when CD40 ligation occurs in the presence of IL4[31,32]. Proliferation has also been seen in B CLL cells in such a system[33] as well as follicle centre lymphoma[34]. B cell precursors

will proliferate when other cytokines are used as well e.g. IL3, IL10[32]. Enhancement of the response is seen with IL3 rather than with IL4 or IL10[32] and combined stimulation of B cell precursors with IL3 and IL10 is similar in terms of growth rate to IL3 alone. It has been suggested that IL3 is acting on more immature cells than IL4 or IL10[32] and indeed maturation is not prominent an IL3/anti-CD40 system.

Mature B lymphocytes have been grown using CD40L rather than stromal cell bound anti-CD40[35] and here the maximal response to IL4 or IL10 is similar when studied for the first 7 days. Ig secretion is regulated by CD40[36] with combinations of cytokines leading to different levels and isotypes of Ig secretion.

The role of CD40 in immune regulation is characterised by its effect on the CD80 (B7) system. CD40 binding by its ligand leads to CD80 (B7.1) and CD86 (B7.2) upregulation[28] two important accessory molecules whose effects will be discussed later. Blockade of CD40L - prevents this upregulation. CD40 has also been demonstrated to be present in Hodgkin and Reed Sternberg (RS-H) cells[37] - this has been demonstrated in RS derived cell lines and in primary H-RS cells. Interestingly, in these cells it did not lead to proliferation or CD80 induction.

In summary, CD40 - via its ligand CD40L or via crosslinking by anti-CD40 Ig leads to clonal expansion, Ig production, isotype switching, and B cell proliferation. These effects can be influenced by exposing the lymphocytes to various cytokines. It is

also expressed in RS-H cells where it may induce cytokine production but does not seem to induce proliferation.

2.3 Cytokines and B Cell Proliferation and Differentiation - IL3, IL4 and IL10

The control of B lymphocyte proliferation and differentiation has been known to be under the control of T cells. Follicle centre lymphomas have been shown to be stimulated to grow under the influence of CD4+ T cell clones[38]. Lymphomas cells expressing t(14;18) cultured in such a way vary in morphology depending on the T cells - some become plasma cell like, others become small with little cytoplasm. T cell contact is required for proliferation and IL2 and IL4 secreted by them appeared important to support growth and differentiation. 2-3 weeks was the maximum period that this would occur for.

IL3

Interleukin 3 - this T cell derived cytokine[39] binds to a receptor with 2 subunits, the alpha subunit is specific for IL3 the beta subunit is shared with various other cytokines[40]. It acts on a wide spectrum of haemopoietic cells[41] - causing the growth of erythroid, myeloid and megakaryocytic lines. It has been used to support patients with chemotherapy induced cytopenia[41]. In B cells its actions are complex, there is evidence that it is a growth factor and that it may cause differentiation[42]. The stimulation is comparable to that achieved with IL4 and differentiation occurs through growth - although some may be via IL6 secretion[42]. The effect of IL3 on CD40 activated B cells is to promote

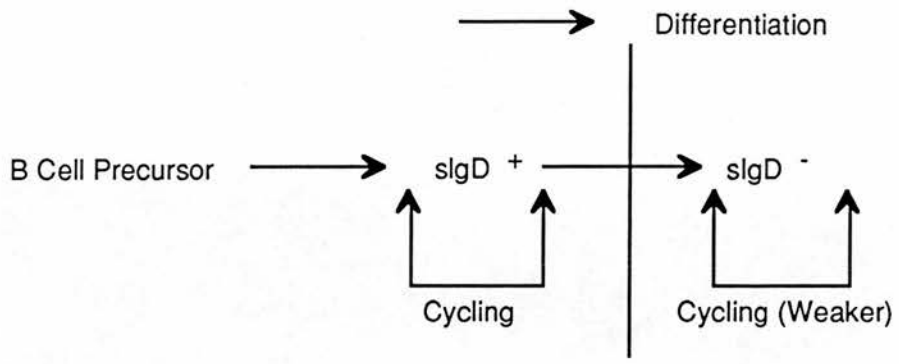
differentiation. Using an anti-CD40 system throughout with Ltk- cells Ig synthesis rises[43].

Normally the IL3 receptor is only transiently expressed, expression may occur in late stage malignant cells e.g. myeloma, follicle centre lymphoma[42]. While it has been found to be a growth factor for follicle centre lymphoma[44] where the IL3 receptor has been found in a cell line derived from such a lymphoma, whose main growth factor was IL3, suggesting that this lymphoma may be retaining characteristics of an earlier stage of development, there is also evidence that IL3 suppresses follicle centre lymphoma growth and that when lymphoma cells are taken from patients and are found to express the IL3 receptor addition of IL3 leads to a reduction in ^{3}H thymidine uptake[45].

IL4

This TH_2 derived cytokine[46,47] elicits responses from macrophages and fibroblasts as well as B cells. B cells, activated either by S. aureus Cowan (SAC), or anti-IgM[48] or anti-CD40[49] will proliferate under its influence. Germinal centre cells alter their morphology[49] whilst normal B cells proliferate without differentiation. $SIgD^+$ blasts proliferate in preference to $sIgD^-$ (activated cells), long term proliferating cells lack germinal central markers, (CD10, CD38) the $SIgD^-$ (see Figure 1) will proliferate for 30 days, the $SIgD^+$ cells for 60 days.

Figure 1: B cell precursor differentiation and proliferation



Some of the SIgD$^-$ pool is renewed by the SIgD$^+$ cells[49]. In malignant culture systems IL4 alone with CLL cells has been found to block apoptosis by upregulating *bcl-2*[50].

IL10

This cytokine - originally termed cytokine synthesis inhibiting factor[51], is a Th_2 derived cytokine with varying effects on the T cells, cells of the monocyte/macrophage series and B lymphocytes.

It is homologous to the open reading frame in EBV, BCRF-1[52,53]. Synthesized by CD4+ T cells, it inhibits the cytokine synthesis by T cells and has an immunosuppressive role[51]. It also inhibits monocyte costimulating activity by inhibiting the upregulation of CD80[54]. In contrast, its effects in B cells tend to be stimulatory. EBV transforms B lymphocytes and proliferative control depends on IL10[55]. Mature B cell lines produce IL10. IL4/anti-CD40 fails to induce IL10. IL10 and IL4 are able separately to induce B cell growth following activation with anti-CD40[56]. However, after 1 - 2

weeks the main effect of IL10 is to induce terminal differentiation, by 21 days - no proliferation occurs[56]. As anti-CD40 has growth promoting activity and IL4 but not IL10 promotes CD40 expression, this may be part of the reason. IL10 is also able to induce proliferation of SAC stimulated cells[57].

Germinal centre cells undergo spontaneous death *in-vitro* via apoptosis[58]. However, IL10 is able to increase *bcl-2* levels and cell viability can be prolonged at 7 days by reducing spontaneous death from 75% to 40%. Germinal centre cells - produce *bcl-2* poorly, neither IL2 nor IL4 can prolong their life. This raises the question as to whether IL10 may play a role in development of follicle centre cell lymphomas following *bcl-2* rearrangement. The differentiating effects of IL10 on B cell blasts grown on marrow stroma has been confirmed[59]. Blocking the effects of endogenous IL10 fails to alter CD40 induced B cell proliferation[56].

Sustained immunoglobulin production occurs (also with IL3). The combination together maximises the effect, the effect on differentiation being independent of the effect on proliferation. CD38 is upregulated and CD20 downregulated and the cells become plasma cell like[59]. Endogenous IL10 is responsible in part for differentiation of B lymphocytes[49]. An EBV lymphoproliferative disease derived cell line produces IL10[60] - interestingly no BCRF-1 is transcribed, therefore autocrine control by hIL10 is suggested.

2.4 Rationale for cytokine combinations in the stromal cell system

Low grade B cell lymphomas, grow very slowly - indeed their growth rate may not be different from benign lymphoid tissue, however, delay in undergoing apoptosis leads to the accumulation of long lived cells[61]. Failure to undergo apoptosis means there is a loss of an important mechanism to keep cell numbers constant[62]. Many attempts to grow low grade lymphoma *in-vitro* have concentrated on achieving cell division[33,34,44], but it is necessary to ensure that the division rate and morphology be retained if the model is to be representative of *in-vitro* conditions.

The *bcl-2* oncoprotein is one of a family of apoptotic suppressor proteins which is upregulated at various stages of normal lymphocyte development[63]. Persistent upregulation may be one important mechanism by which malignant B cells retain their advantage over their benign counterparts[62]. It is important in lymphoma expressing *bcl-2* that this expression continue during *in-vitro* culture.

The cytokines described above are unable to provide the desired effect of slow growing, mature, malignant lymphocytes and combinations of cytokines with predominant proliferating or differentiating activity yield improved results. The role of T cells in regulating B cell growth might be utilised so long as they did not overgrow the B cells.

The following systems were investigated: (1) IL4/anti-CD40 - all its effects on growth and phenotype are already described[34] and (2) IL3/IL10 anti-CD40.

The relative success of the IL3/IL10 anti-CD40 stromal cell system in culturing low grade B cell lymphoma and the realisation that there is evidence that Hodgkin's lymphoma is B cell derived at least in some cases prompted the investigation of this system in Hodgkin's lymphoma as well.

CHAPTER 3

THE STROMAL CELL CULTURE SYSTEM

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3.1 Introduction

This chapter describes the setting up of a stromal cell system for the primary culture of low grade B cell and Hodgkin's lymphoma. The rationale for the system, and the various cytokines used are described as is the preparation and storage of the lymphoma tissue. Techniques to verify the nature of the cultured cells are explained and comparison is made with the culture of benign lymphoid tissue.

3.2 List of tumours and benign lymphoid tissue taken for culture

Non-Hodgkin's Lymphoma

Tumour	Histology (REAL) Classification[5]
L2	Follicle centre lymphoma, follicular, grade 1 (FCL)
L3	B-cell small lymphocytic lymphoma (SLL)
L4	Follicle centre lymphoma, follicular, grade 1
L5	Follicle centre lymphoma, follicular, grade 1
L6	Splenic marginal zone B cell lymphoma with areas of transformation to diffuse large B cell lymphoma (SMZL/DLBCL)
L7	Follicle centre lymphoma, follicular, grade 1
L8	Follicle centre lymphoma, diffuse, grade 1
L9	B-cell small lymphocytic lymphoma
L10	Follicle centre lymphoma, follicular, grade 1
L11	Follicle centre lymphoma, follicular, grade 1
L12	Follicle centre lymphoma, follicular, grade 3
L13	Follicle centre lymphoma, follicular, grade 1

Hodgkin's Lymphoma

L17 - Lymphocyte predominant (LP)

L18 - Mixed cellularity (MC)

L20 - Lymphocyte predominant with a single area of transformation to diffuse large B cell lymphoma

L25 - Mixed cellularity

L27 - Nodular sclerosing (BNLI grade 2) (NS)

L28 - Nodular sclerosing (BNLI grade 1)

Benign Lymphoid Tissue

R1 - Benign reactive lymph node.

T1 - Tonsil removed at tonsillectomy.

3.3 Preparation of cells for culture

The lymph nodes and tonsils studied were all prepared similarly. Samples were taken at biopsy or in the case of the tonsillar control at tonsillectomy. The samples were processed in a laminar flow hood. The tonsils were sprayed using 70% ethanol preceding dissection. The tonsil/lymph node was then dissected in a RPMI based medium (RPMI base) consisting of RPMI 1640 400ml with glutamine, Fetal calf serum (Gibco, Paisley, Scotland) 40ml, Hepes 25mmol/L (Gibco), Penicillin and Streptomycin solution (Gibco) 5ml.

A cell suspension was prepared and this was centrifuged at 600g for 5 minutes at 25°C before washing in RPMI base a further two times. The solution was now ready for centrifugation on Ficoll Hypaque (Lymphoprep, Nycomed Pharma, Oslo, Norway) at 1000g for 25 minutes at 25°C. The mononuclear fraction was removed and washed twice. The pellet was then resuspended in 10ml RPMI base, and a cell count was carried out manually using a haemocytometer. Base line immunophenotyping was then carried out. The cells were then spun down and a freezing mixture consisting of RPMI 70ml, Fetal Calf Serum 20ml, Dimethylsulphoxide (DMSO) 10ml (Fluka) was added dropwise to ensure a final concentration of 2×10^7 cells/ml. The cells were then vortexed and aliquoted into 1ml Nunc freezing tubes. These

were put on dry ice for 40 minutes before placing in liquid nitrogen. The vials were numbered according to the tumour and each individual vial had a two digit identification number.

e.g. L2 07
↑ ↑
tumour 7th tube

3.4 Preparation of fibroblast monolayer

The stromal cells used were mouse fibroblast Ltk- cells, transfected with FcγrII receptor-CD32[64], which is the monocyte Fc receptor. The receptor has 61% homology with the murine IgG_1 receptor. The stromal cells were grown in Falcon 100ml flasks (Beckton Dickinson) in a HAT medium, to ensure that the growing fibroblasts did express the Fc receptor. The medium used consisted of RPMI 1640 with glutamine 450ml, Fetal Calf Serum 50ml (10% $^{vol}/_{vol}$), HAT (1:200, Sigma).

The cells grew as an adherent monolayer; on reaching confluence, the medium was aspirated and they were freed using 2 millimole/litre EDTA, at 37°C for 5 minutes. The cells were then aspirated and centrifuged at 600g, 25°C for 5 minutes. They were then resuspended in 48ml of the HAT medium and vortexed vigorously. 1ml was placed in each well of a 24 well culture plate (Beckton Dickinson). The cells were then allowed to grow to 80-90% confluence at 37°C with 5% $C0_2$, before been irradiated to a dose of 75Gy. Following irradiation, growth did not stop immediately and indeed the monolayer often became confluent

over the next 48-72hrs. The Fc receptor would act as anchor for anti-CD40 - essential for establishing lymphocyte proliferation.

3.5 Resuspension of lymphoma cells for culture

These were initially resuspended in RPMI base medium in which they were washed twice to remove DMSO. The cells were then resuspended in a holding medium of Dulbecco's without phenol red (Gibco) with supplemented bovine serum albumin 10g/L (Sigma) and human immunoglobulin 0.5g/L (Sandoglobulin, Sandoz). The cells were left in this medium for a maximum of 8hrs at 4°C. Viability was assessed by 0.1% trypan blue (Sigma) exclusion. It was usual to discard vials with viabilities of less than 70%.

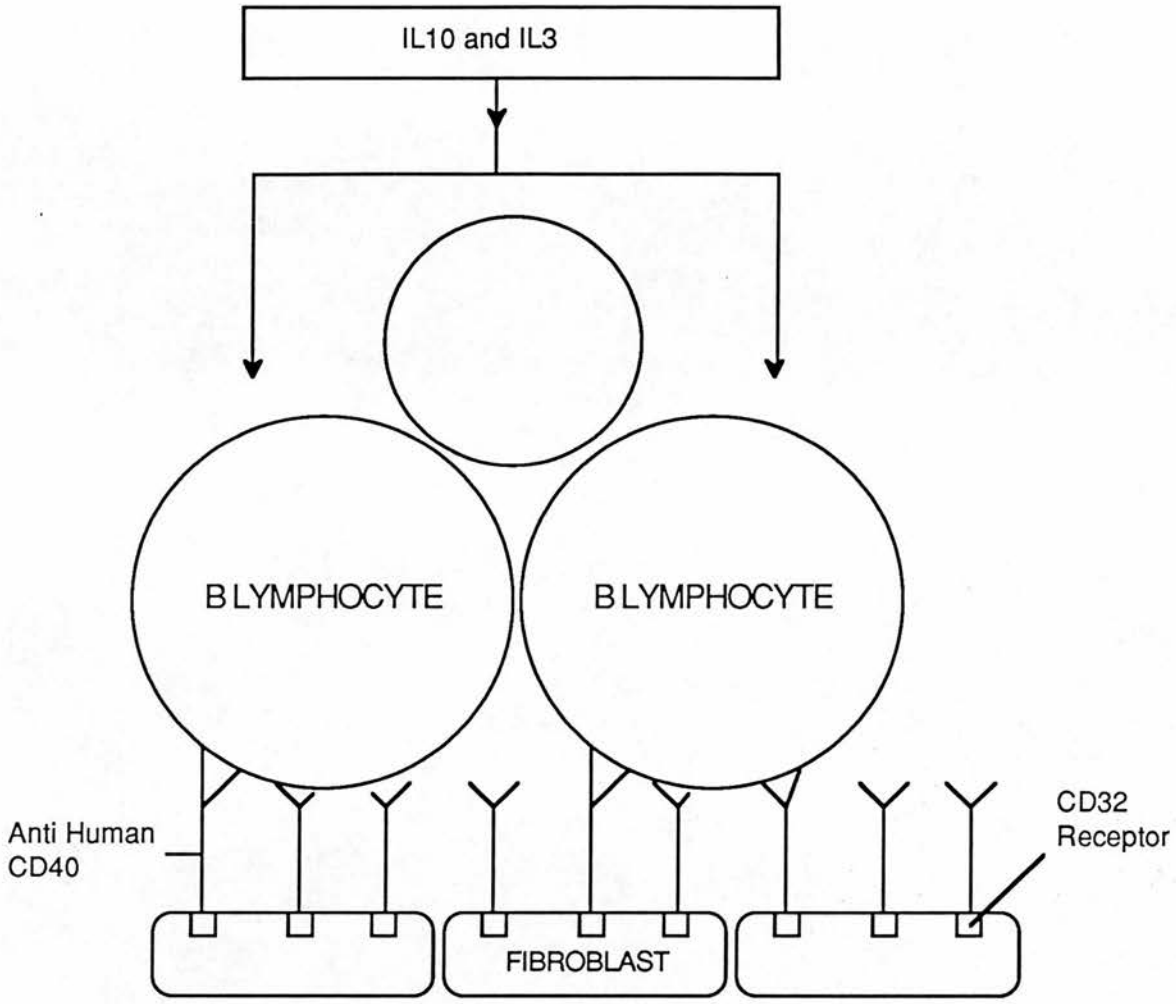
The irradiated fibroblasts were taken in their 24 well plates and the supernatant (containing HAT) was aspirated and 1-2ml of RPMI base medium put in each well. This was then aspirated prior to the plating out of the lymphoma cells. The lymphoma cells were counted and resuspended to ensure a viable cell count of 5 x 10^5/ml in a growth medium consisting of Iscove's modified Dulbecco's medium without FCS (Gibco) with 50mg/L human holotransferrin, bovine serum albumin 5g/L; bovine insulin 5mg/L; long chain fatty acids (cis 9 octadecenoic, all cis 9, 12, octadecadienoic, and hexadecanoic acid all at 1mg/L) (all from Sigma, FCS 20ml/L ($^{vol}/_{vol}$), penicillin and streptomycin (Gibco), Gentamicin 40mg/L (Roussel) and Amphotericin 2.5mg/L (Squibb) anti-CD40 1mg/L (Serotec) and various cytokines IL3 20µg/L (kindly provided by Amgen Inc., Thousand Oaks, California); IL4

46μg/L (kindly provided by Schering Plough Research, Bloomfield, New Jersey) and IL10 100μg/L (kindly provided by Amgen Inc., Thousand Oaks, California).

Usually 8 x 10^6 lymphoma cells were taken suspended in 16ml of growth medium with supplemented cytokines and anti-CD40 and pipetted into 8 wells (2ml per well; 10^6 cells per well).

Depending on their speed of growth the wells had 1ml of growth medium aspirated at day 7-10 and fresh medium with cytokines supplemented but this time only 0.5mg/L CD40 was added. After 14 days the cells were gently pipetted, washed and resuspended in fresh medium to plate down on a new monolayer.

FIGURE 1: THE IL3/IL10 ANTI-CD40 STROMAL CELL CULTURE SYSTEM



3.6 Depletion of T and virgin B lymphocytes

In some experiments it was decided to deplete the cell suspensions of T lymphocytes and sometimes the IgD+ B lymphocytes as well - this was the case to where CD80 expression was being induced using IL4/anti-CD40.

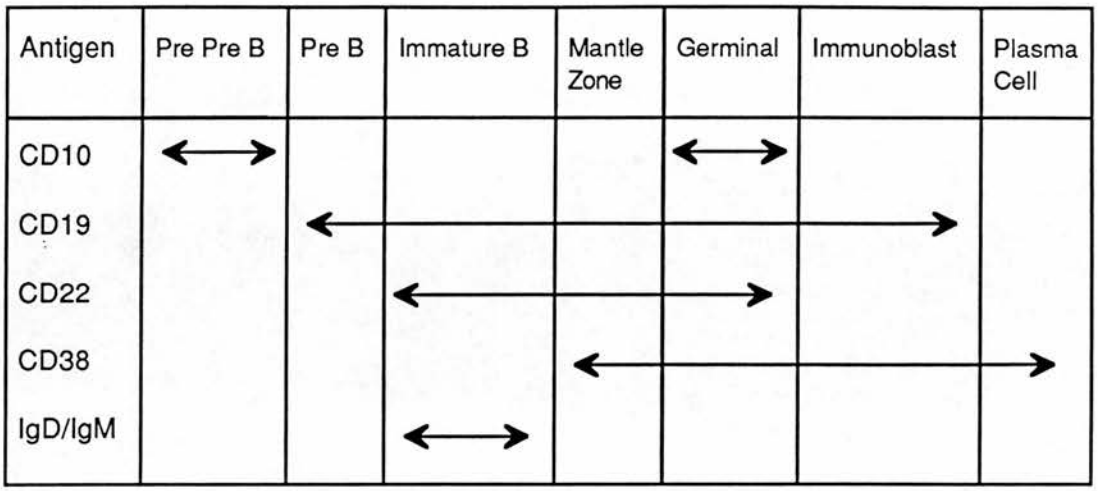
2 x 10^7 lymphoma cells were taken prior to culture and were incubated with monoclonal anti-IgD at a concentration of

100µl/1ml/10^7 cells, anti-CD8 50µl/1ml/10^7 cells and anti-CD4 250µl/1ml/10^7 cells (all Dako). The amounts of antibody used were chosen following titration of serial dilutions of the antibodies with tonsillar lymphocytes. After 30 minutes incubation at 4°C, the cells were washed twice to remove any unbound antibody, and Dynabeads (Dynel International, Oslo, Norway) which were coated with anti-mouse Ig and had themselves been washed in PBSA to remove any azide preservative, were incubated together at a concentration of 1ml/10^7 cells on a rotating mixer at 4°C for 1hr. The cells were then removed using magnetic separation and immunophenotyped as appropriate.

3.7 Phenotyping of cells and cell sorting

Cells were immunophenotyped as a baseline prior to culture. Antibodies were added to cells for 30 minutes at 4°C in 96 well plates and then centrifuged at 600g for 1 minute at 4°C, they were then resuspended in 100µl PBSA prior to re-centrifugation, before fixing in 250µl of 1% paraformaldehyde. CD30 based cell sorts were carried out by taking 2 x 10^7 cells of cultured Hodgkin's lymphoma on day 15 and incubating them with 200µl of anti-CD30 FITC (Dako), the cells were then sorted using a FACStar Plus cell sorter with a fluorescence histogram for CD30 FITC (fluoroscein isothiocyanate) being produced and a marker set against a negative control. Those cells with fluorescence above a certain threshold were deemed CD30+ve and collected as such, with the CD30-ve cells collected separately.

Figure 2: Diagrammatic Representation of B cell Antigen Expression - only showing antigens studied *in-vitro* culture



As can be seen it possible to place the main population of cells by the combined expression of various antigens e.g. if a majority of cells express CD10, CD19, CD22 and CD38 they are likely to be at the germinal centre stage of development, whilst those expressing CD19 and CD22 to a lesser extent, and lacking CD10 are likely to be less mature.

3.8 Preparation of cells for *bcl-2* staining

As this oncoprotein is found at various sites within cells, permeabilization of the membrane is required. Temporary permeabilization was achieved using the detergent saponin (Sigma)[65]. The cells were suspended in 0.3% saponin in phosphate buffered saline (PBSA) for 15 minutes at 25°C. They were then phenotyped using anti-*bcl-2*-FITC (Dako) (10µl/10^6/100µl) in 0.1% saponin solution for 30 minutes at 25°C. The cells were then centrifuged and resuspended in PBSA with 1% paraformaldehyde. The negative control (IgG1, FITC negative

control, Dako) was prepared similarly for this analysis 5,000 events were surveyed.

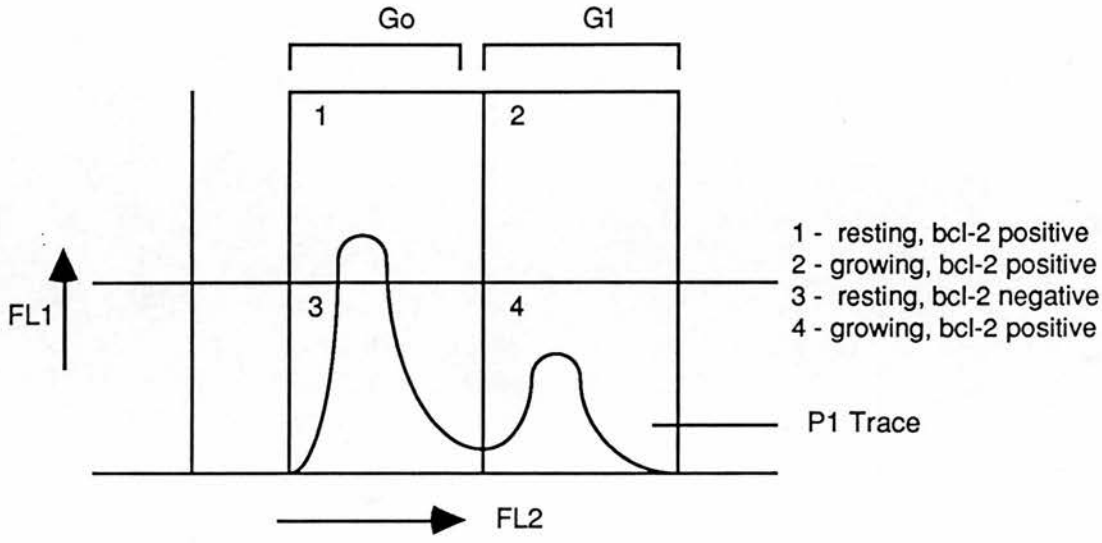
3.9 Growth Fraction Assessment

Cells were fixed in 70% ethanol. Ribonuclease A type 1 (Boehringer Mannheim) 100k units/ml was added. Propidium iodide (PI) 50μg/ml was then introduced. PI fluorescence was detected at over 580nm. Cells from the fibroblast monolayer were gated out using forward and side scatter. 2,000 cells were analysed. Cells with PI fluorescence in the hypodiploid and diploid G_0/G_1 peak and the hyperdiploid S + G2 (growth fraction) were determined on red fluorescence histograms using LysysII software.

3.10 Bcl-2 in growing and resting cells (see Figure 3)

Data were displayed using a quadrantic plot:- X axis - FL-1 channel (FITC) (ie: *bcl-2*), Y axis FL-2 (ie PI). Markers were placed between the growing and resting cells based on PI staining and between those that did and did not express *bcl-2* (by comparison with IgG1, FITC - negative control). It was possible to separate the following into different quadrants - resting cells - *bcl-2* - negative (3), resting *bcl-2* positive (1), growing *bcl-2* negative (4), growing *bcl-2* positive (2).

Figure 3: Quadrantic plot showing bcl-2/growth fraction relationship



3.11 Kappa and Lambda light chain staining

Cells were taken and not blocked with human immunoglobulin, if they had been they were put in RPMI 1640 at 37°C for 1 hour first. 2 colour fluorescence was used. Polyclonal $F(ab)_2$ antibodies were used (Dako). They were diluted to 1µl/10^6/100ml κ-FITC, λ-PE, incubated for 20 minutes with the cells (both antibodies simultaneously).

3.12 Morphological Examination

Cytocentrifugation specimens were prepared. Cells were harvested from the culture wells and resuspended in PBSA with 0.2% albumin. 100ml of cell suspension containing 10^5-10^6 cells were cytocentrifuged in a Cytospin 3 at 600rpm for 5 minutes. The slides were then air dried before staining using the May

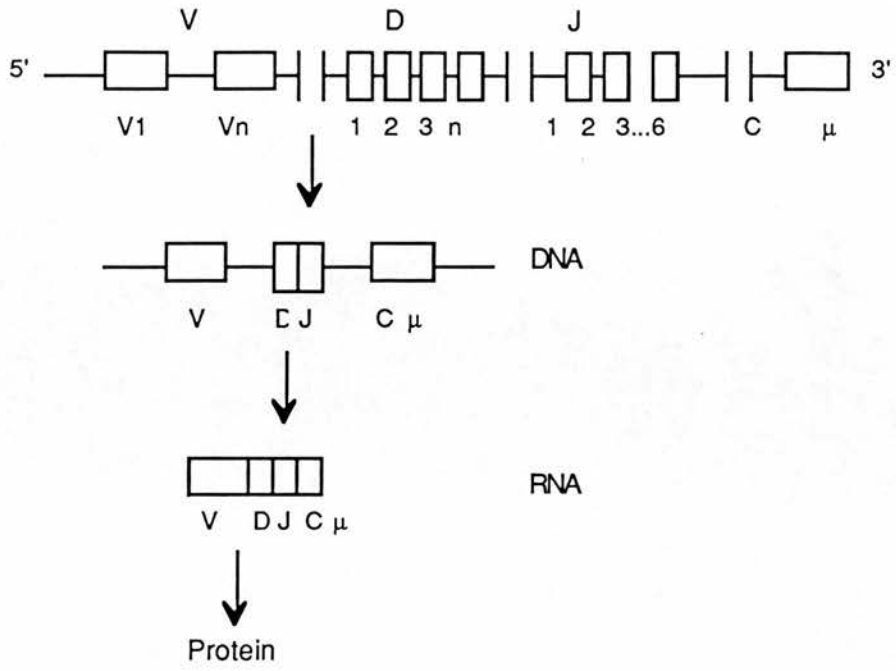
Grunwald Giemsa Method. They were then examined under light microscopy.

3.13 Synthesis of Immunoglobulin

Immunoglobulin synthesised may be confined to the cytoplasm, expressed on the surface membrane, or secreted. In the germline state, κ and λ light chain genes each exist as a number of variable (V), joining (J) segments and a constant (C) region[66]. During B cell development one of the V regions is juxtaposed to one of the J regions with excision of the intervening DNA. The rearranged VJ segment together with the C region is transcribed. Intervening RNA is spliced out to form the final mRNA. Both or only one allele may rearrange.

Heavy chain rearrangement is complicated by the fact that there are diversity (D) segments between the V and J regions, DJ joining occurs first, followed by C-DJ joining.

9 genes correspond to the different Ig subtypes. Class switching occurs when a rearrangement within this H chain gene cluster inserts a new constant gene instead of Cμ; this allows the antigenic determinant to be maintained despite a change in Ig class. Immunoglobulin rearrangements occur in a set order: Ig heavy chains followed by κ light chains, followed by λ light chains. Therefore if a λ Ig is secreted all genes will have rearranged, although the κ rearrangement will be non-productive[66].



Rearrangement of the Ig Heavy Chain[66]

In B lymphocytes, the V_H region contains 3 hypervariable complementary determining regions - CDRI, II and III and their relatively conserved framework regions FRI, II and III. Rearrangement brings FRII and the CDR regions[67] into close proximity allowing a polymerase chain reaction based technique to look for monoclonal IgH rearrangements. If the two regions are in the germline state they remain too far apart making generation PCR IgH products impossible. Such a technique was used to study the clonal origin of Hodgkin's lymphoma in culture (described later).

3.14 PCR based technique for the heavy chain rearrangement - (Hodgkin's Lymphoma)

Cultured cells were washed twice in PBSA, and DNA was prepared by phenol/chloroform extraction, following detergent lysis and proteinase k digestion. The presentation samples had DNA either extracted from paraffin blocks or from cell suspensions taken at biopsy.

A highly sensitive semi-nested PCR was performed in a Gene-Amp PCR system 9600 (Perkin Elmer Cetus, Norwalk CT). The first amplification was performed using an upstream consensus primer FR2A[67,68] designed from homologous regions within V_H framework II and a downstream primer LJH that bound to all published J_H gene segments. For the initial amplification, 0.5µg of genomic DNA was used. For the reamplification, the downstream primer LJH was replaced by a nested consensus primer VLJH, with 1% of the initially amplified product being used as a template. The PCR conditions comprised five initial high stringent cycles at 96°C for 15 seconds for DNA denaturation, at 63°C for 30 seconds for primer annealing and at 72°C for 30 seconds for primer extension, followed by 35 cycles with the same denaturation and extension conditions but with an annealing temperature of 57°C. The reamplification consisted of 25 cycles of denaturation at 96°C for 15 seconds, annealing to 63°C for 30 seconds and extension at 72°C for 30 seconds. The differences beside the total number of cycles, the primer usage and highly stringent annealing temperature between the first and reamplification, were the concentrations of $MgCl_2$ (1.5mmol/L

versus 2.0mmol/L) and the amount of each primer (first amplification with 400ng FR2A, 100ng VLJH; reamplification with 200ng FR2A and 200ng VLJH).

10µl of each amplified product were applied to a 2% agarose gel and visualised by staining with ethidium bromide.

3.14.1 *DNA sequence analysis*

The sequencing of the PCR product was performed using an automated DNA sequencer (Applied Biosystems 373A) by using the Dye Deoxy Terminator Method for use solely with this system.

The PCR products were passed through a Sephadex G50 column and after ethanol precipitation the products were sequenced in both directions using the FR2A and VLJH primers respectively. Only cases with a complete homology between both sequences were chosen for comparison with published VJH germ line sequences. To act as controls, DNA was extracted from mouse fibroblasts which provided the monolayer, and cultured benign lymphocytes from a reactive role.

3.15 Preparation of cells for Cytogenetic Analysis (Hodgkin's Lymphoma)

Cells were taken at day 10-15 in culture for cytogenetic analysis. Whilst still adherent to the fibroblast monolayer they were treated with colcemid (Gibco) 10µg/ml for 16hrs before pipetting centrifugation at 600g for 5 minutes at 20°C. The resultant pellet was resuspended in 75mmol/L KCL and then incubated for 10

minutes at 37°C. This was then centrifuged at 600g for 5 minutes and fixed in 3:1 methanol:glacial ethanoic acid, centrifuged and resuspended in this a total of 3 times. Slides were prepared by placing a drop of fixed material on a slide, and then left to age for several days at room temperature and banded using 0.1% trypsin (Difco, Detroit, USA) for 5-10 seconds and stained with Giemsa for 1 minute prior to drying and analysis.

3.16 EBV Status and Light Chain Restriction (Hodgkin's Lymphoma)

Paraffin sections of all lymph node biopsies were stained using a streptavidin biotin peroxidase method after heat pretreatment using monoclonal antibody against latent membrane protein (LMP) of EBV (Dako). Both cases of LP-HL were stained using polyclonal antibodies to Kappa and Lambda light chains after trypsin digestion.

CHAPTER 4

CULTURE OF BENIGN LYMPHOID TISSUE USING THE IL3/IL10 STROMAL CELL SYSTEM

4. CULTURE OF BENIGN LYMPHOID TISSUE USING THE IL3/IL10 STROMAL CELL SYSTEM

4. CULTURE OF BENIGN LYMPHOID TISSUE USING THE IL3/IL10 STROMAL CELL SYSTEM

4.1 Introduction

This chapter examines the effect of long term culture on benign lymphocytes using the IL3/IL10 stromal cell system. Two sources of lymphoid tissue are used and sequential analyses of flow cytometric and morphological data are presented.

4.2 Phenotyping benign lymphoid tissue

Table 1: Table of Phenotyping for IL3/IL10 anti-CD40 - Benign Lymphoid Tissue.

Antibody	Day 1	Day 5	Day 10	Day 15	Day 20	Day 30
IgG_1 FITC (Con) (Dako)	•	•	•	•	•	•
CD3 FITC (Dako)	•	•	•	•	•	•
CD10 FITC (Dako)	•	•	•	•	•	•
CD14 FITC (Dako)	•		•		•	•
CD19 FITC (Dako)	•	•	•	•	•	•
CD22 FITC (Immunotech)	•	•	•	•	•	•
CD38 FITC (Immunotech)	•	•	•	•	•	•
IgD $F(ab)_2$ (Dako)	•		•		•	•
κ FITC F(ab)2 (Dako)	•		•		•	•
λ PE $F(ab)_2$ (Dako)	•		•		•	•
CD15 FITC (Dako)	•		•		•	•
CD30 FITC (Dako)	•		•		•	•
Growth Fraction (PI)	•	•	•	•	•	•

Cells were taken from two different sources (1) a tonsil (2) a reactive lymph node. No attempt was made to deplete the T cells. The cells were grown for 30 days and were still vigorously dividing when the cultures were discarded at this point. Replating onto new fibroblasts was carried out at day 15. Cells were taken for

cytocentrifugation on days 10, 15, 20 and 30. Growth fraction was assessed at 5 day intervals. Immunophenotyping was carried out every 5 days - see Table 1. A marker was set based on the negative control, percentages are given compared to this.

4.3 Results

Table 2: Immunophenotyping Results - Tonsil (T1)

Antibody	Day 1	Day 5	Day 10	Day 15	Day 20	Day 30
G_1	4	2	1	0	2	2
CD3	35	13	13	12	19	13
CD10	14	3	3	5	6	4
CD14	9	-	2	-	3	3
CD19	67	75	45	66	47	64
CD22	59	69	59	61	44	20
CD38	38	9	1	1	1	7
IgD	30	-	40	-	12	19
κ/λ	Both	-	Both	-	Both	Both
CD15	23	-	15	-	24	18
CD30	6	-	28	-	33	31
GF	14	38	17	21	16	14

Table 3: Immunophenotyping Results - Reactive Node R1

Antibody	Day 1	Day 5	Day 10	Day 15	Day 20	Day 30
G_1	1	0	0	0	2	2
CD3	60	26	18	21	45	35
CD10	15	1	2	0	3	4
CD14	7	-	2	-	1	27
CD19	44	69	35	48	15	34
CD22	38	69	68	57	21	3
CD38	19	2	2	4	6	3
IgD	19	-	31	-	6	2
κ/λ	Both	Both	Both	Both	Both	Both
CD15	19	-	11	17	-	1
CD30	4	-	11	9	-	1
GF	13	38	17	23	25	10

The immunophenotyping confirmed a B-cell expansion. There was no evidence of T cell (as judged by CD3) or monocyte (as judged by CD14) overgrowth suggesting that depletion of these is unnecessary in this system. Overall, the percentages remained fairly constant and close to the in-vivo situation (day 1). It was disappointing to see the loss of the germinal centre cell phenotype (CD10, CD38). In both cases loss of these cells within the first 5 days was not accompanied by their return later in culture.

<u>CD19 and CD22</u> - CD22 is a more restricted B-cell marker than CD19. Both remained throughout culture but both diminished after a surge in the first 10 days although evidence of a rise in CD19 occurred at day 30. IgD positive cells fell after an initial surge - it did not appear that any IgD^+ cells were proliferating as

has been suggested as the growth fraction remained high in the last 15 days despite the disappearance of IgD bearing cells.

κ and λ – No evidence of light chain restriction occurred nor would it be expected in benign tissue.

CD15 and CD30 - These two markers were studied as they were present Reed Sternberg - Hodgkin's cells[69,70] and it was important to know if they appeared in benign lymphoid tissue in culture.

CD15 - A granulocyte marker, not present in macrophages or lymphocytes or platelets, but seen in promyelocytes and myelocytes.

CD30 - (Ki-1)[24,70] - A membrane protein seen in RS-H and a varying proportion of activated T and B cells. It is seen in RS-H cells and anaplastic large cell lymphomas.

In the reactive node, there was no suggestion of upregulation during culture, however, in the tonsil CD30 did upregulate to a degree this may have represented activation by CD40. The high initial expression of CD15 is more difficult to explain.

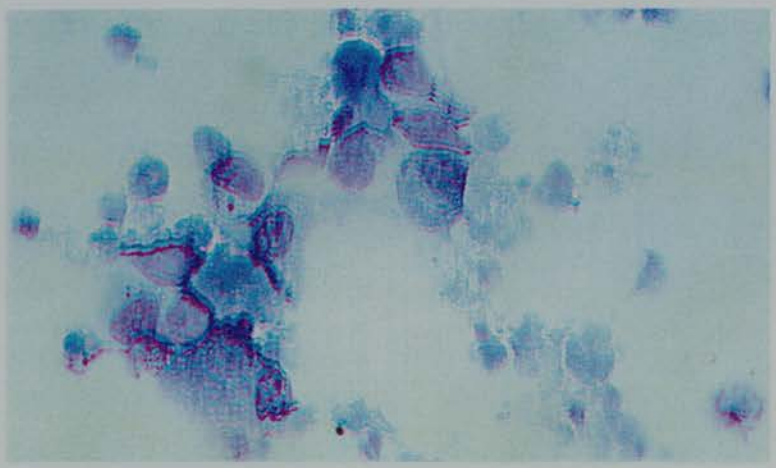
Growth Fraction - This remained high in both samples generally exceeding the initial growth fraction.

4.4 Cytocentrifugation Specimens

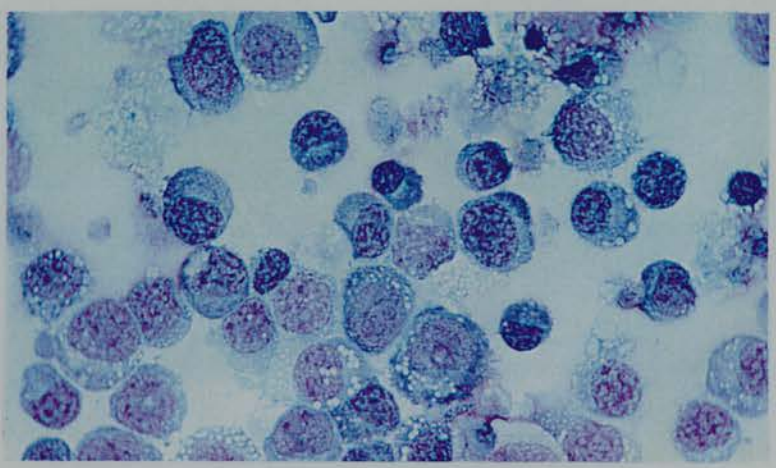
Blast cells and centrocytes were present from day 10. Reduced numbers of blast cells were present by day 20 and numerous plasma cells with increasing cytoplasm were seen from day 20. This is in line with the differentiating effects of IL10 (see Prints 1 - 6).

Prints 1 - 3: **Cytocentriguation specimens from a reactive node: Day 9, 15 and 30.**

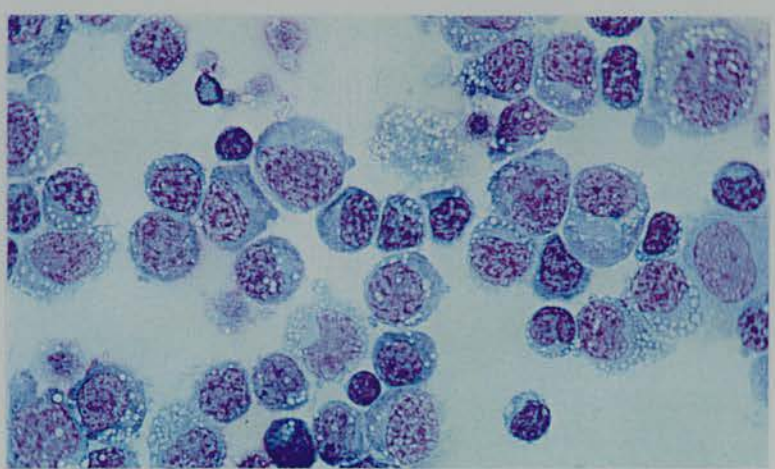
Day 9



Day 15



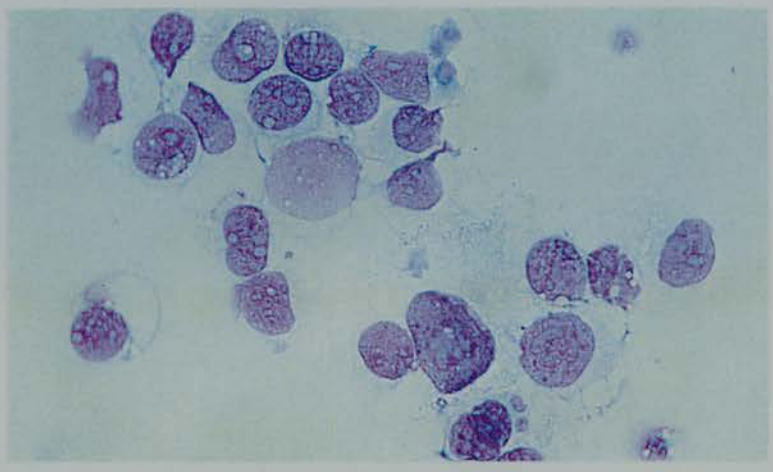
Day 30



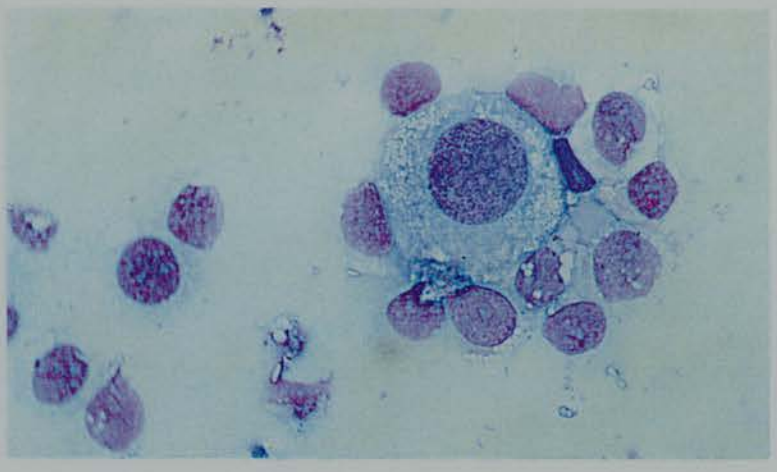
Prints 4 - 6: **Cytocentriguation specimens from a tonsil:**

Day 9, 15 and 30.

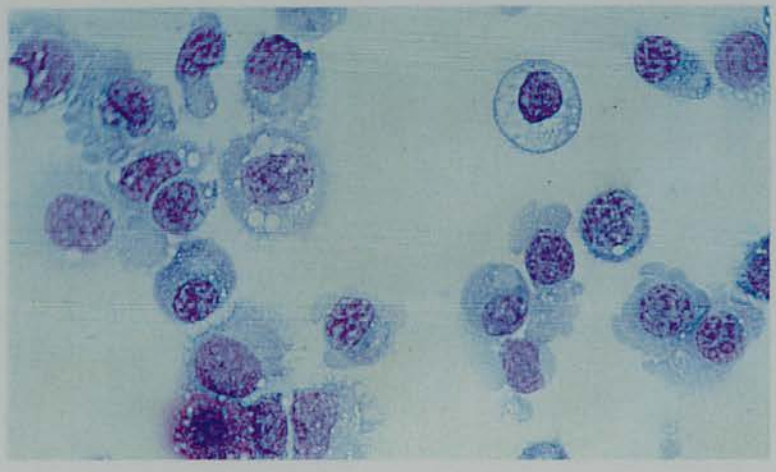
Day 9



Day 15



Day 30



LEGENDS FOR PRINTS OF BENIGN LYMPHOCYTES IN CULTURE

Prints 1 - 3: Cells cultured from reactive node (R1) shown at day 9 (Print 1), day 15 (Print 2) and day 30 (Print 3). The cells show increasing plasma cell differentiation becoming larger with increasing amounts of cytoplasm.

Prints 4 - 6: Cells cultured from a tonsil shown at day 9 (Print 4), day 15 (Print 5) where they can clearly be seen clumped around a central mouse fibroblast and day 30 (Print 6). Again plasma cell differentiation is prominent.

Table 4: Cytocentrifugation specimens

Sample	Day 9	Day 15	Day 20	Day 30
R1	Large numbers of blast cells (50%). Smaller numbers of centrocytes. Scattered small lymphocytes.	Blast cells (40%). Centrocytes (45%). Small numbers of plasma cells and lymphocytes.	Blast cells (30%). Centrocytes (50%). Plasma cells (10%).	
T1		Blast cells (35%). Centrocytes (55%). Rare plasma cells and lymphocytes.	Blast cells (15%). Centrocytes (70%). Plasma cells and lymphocytes.	Blast cells (7%). Centrocytes (70%). Plasma cells (20%).

4.5 Summary

The IL3/IL10 CD40 stromal cell system supports lymphocyte culture for at least 30 days. The proliferation is predominantly B cell with no evidence of light chain restriction. Morphology shows increasing numbers of plasma cells and centrocytes with reducing numbers of blast cells. The germinal centre cell phenotype is lost rapidly. There is little upregulation of CD15 and CD30.

CHAPTER 5

CULTURE OF LOW GRADE B CELL LYMPHOMA USING THE IL3/IL10 STROMAL CELL SYSTEM - COMPARISONS WITH IL4/ANTI-CD40

5. CULTURE OF LOW GRADE B CELL LYMPHOMA USING THE IL3/IL10 STROMAL CELL SYSTEM - COMPARISONS WITH IL4/ANTI-CD40

5. CULTURE OF LOW GRADE B CELL LYMPHOMA USING THE IL3/IL10 STROMAL CELL SYSTEM - COMPARISONS WITH IL4/ANTI-CD40

5.1 Introduction

8 Low grade B cell lymphomas were studied in depth - 5 follicle centre cell lymphomas, 2 B cell small lymphocytic lymphomas, 1 splenic marginal zone lymphoma with areas of transformation to diffuse large B cell lymphoma.

The lymphomas were grown for 20-23 days - immunophenotyping, growth fraction, cell numbers, cytocentrifugation specimens for morphology were carried out at regular intervals. For analysis results were spilt between the follicle centre lymphomas and the non-follicle centre cell lymphomas. Growth fractions were separated between those lymphomas which showed evidence of transformation to high grade histology on biopsy prior to culture and those that did not.

The results from lymphomas grown in the IL4/anti-CD40 system were taken from samples that had their T cell and IgD bearing B cells removed, however when compared to those which did not; no substantial difference in GF could be determined.

5.2 Immunophenotyping

Table 1: Immunophenotyping of cells

IMMUNOPHENOTYPING OF CELLS					
ANTIBODY	**DAY 1**	**DAY 5**	**DAY 10**	**DAY 15**	**DAY 23**
IgG_1, FITC Mouse Negative Control (Dako)	•	•	•	•	•
CD3 FITC (Dako)	•	•	•	•	•
CD10 FITC (Dako)	•	•	•	•	•
CD14 FITC (Dako)	•		•		•
CD19 FITC (Dako)	•	•	•	•	•
CD22 FITC (Immunotech)	•	•	•	•	•
CD38 FITC (Immunotech)	•	•	•	•	•
IgD $F(ab)_2$ FITC Polyclonal (Dako)	•		•		•
Kappa $F(ab)_2$ PE Polyclonal (Dako)	•		•		•
Lambda $F(ab)_2$ PE Polyclonal (Dako)	•		•		•
Anti *bcl-2* FITC (Dako)	•		•		•
CD34 FITC (Beckton-Dickinson)					•

5.3 Histology (see Prints 1-8) - in IL3/IL10 anti-CD40 system

Cytospins were examined at days 15 and 23 and compared to touch imprints made of the original biopsies. (See Table 2).

Follicle Centre Lymphomas (L2, L5, L10, L12)

Three of the follicle centre lymphomas maintained their parent morphology with relatively plentiful centrocytes and rare

centroblasts. Tumour cells were often adherent to the mouse fibroblasts. 2 cases showed an overgrowth of large nucleolated blast cells. 1 of these cases had plentiful blasts on the parent imprint while the other case had an initial morphology indistinguishable from the 3 non-transformed cases. By day 23 there was relatively plentiful cell debris and cells showed progressive cytoplasmic vacuolation in culture.

Small lymphocytic and splenic marginal zone lymphomas (L3, L6, L9)

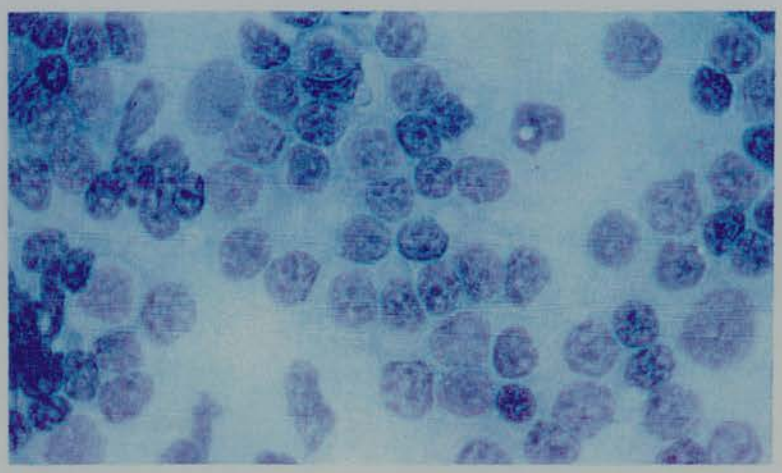
Cells from the L3 case of B-small cell lymphocytic lymphoma retained the morphology of small lymphocytes throughout the culture period. The splenic marginal zone lymphoma which had immunoblasts (transformed component) on a back round of lymphoplasmacytoid cells - maintained this composite pattern. Culture L9 - a small B cell lymphocytic lymphoma with lymphoplasmacytoid differentiation continued to maintain these cells in culture.

Table 2: Morphology of cultured cells

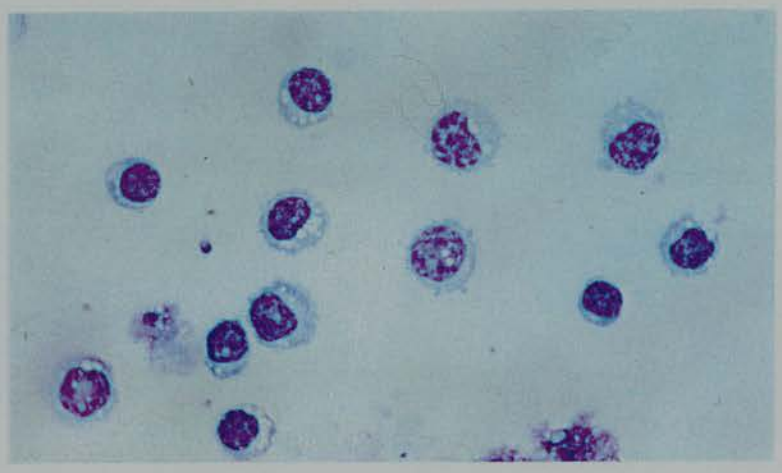
MORPHOLOGY OF CULTURED CELLS CYTOCENTRIFUGATION SPECIMENS			
NO	**LYMPHOMA REAL CLASSIFICATION**	**DAY 15**	**DAY 23**
L2	Follicle centre lymphoma, follicular, grade 1	Some centrocytes clusters of vacuolated blasts	Large blasts with plentiful cytoplasm
L3	B cell small lymphocytic lymphoma	Lymphocytes with increased cytoplasm	As day 15
L5	Follicle centre lymphoma, follicular, grade 1	Small clumps of centrocytes prominent nucleoi	Insufficient cells
L6	Splenic marginal zone B cell lymphoma with areas of transformation to diffuse large B cell lymphoma	Clumps of immunoblasts and lymphoplasmacytoid cells	As day 15 but larger nucleoi seen in blast cells
L9	B cell small lymphocytic lymphoma	Lymphoplasmacytoid cells with vacuolation	As day 15
L10	Follicle centre lymphoma, follicular, grade 1	Centrocytes	Centrocytes with vacuolation
L11	Follicle centre lymphoma, follicular, grade 1	Large centrocytes rare blasts	Centrocytes with vacuolation
L12	Follicle centre lymphoma, follicular, grade 3	Large numbers of blasts	Large clusters of blasts with some centrocytes

Prints 1 - 2: **Touch imprints and cytocentrifugation specimens of low grade B cell lymphomas studied**

L3 Touch

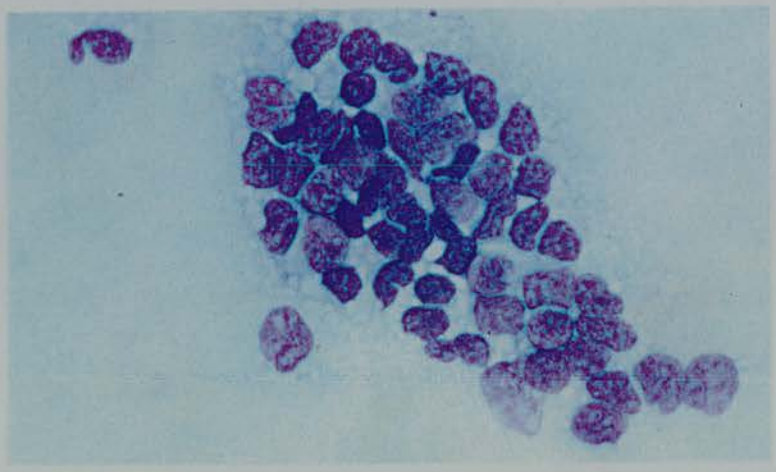


L3 Culture

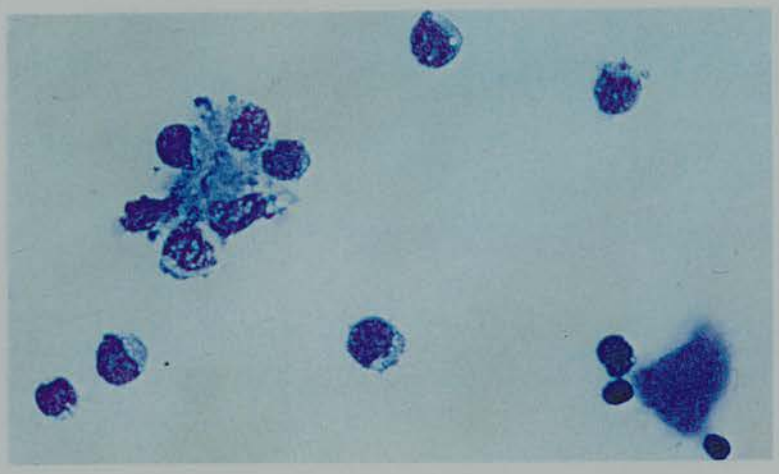


Prints 3 - 4: **Touch imprints and cytocentrifugation specimens of low grade B cell lymphomas studied**

L5 Touch

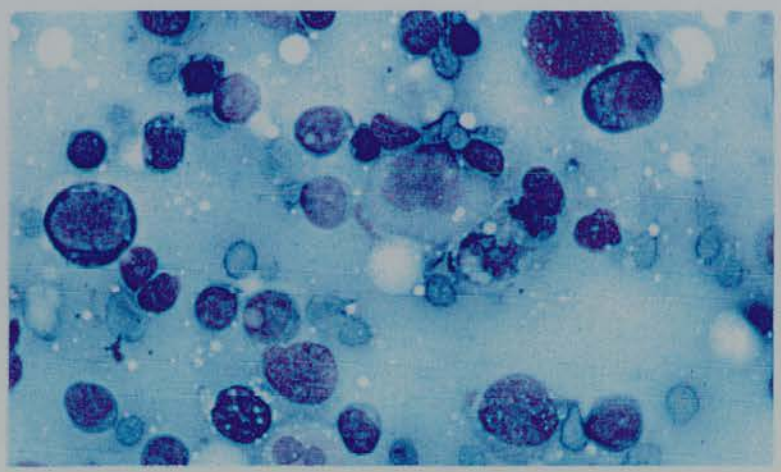


L5 Culture

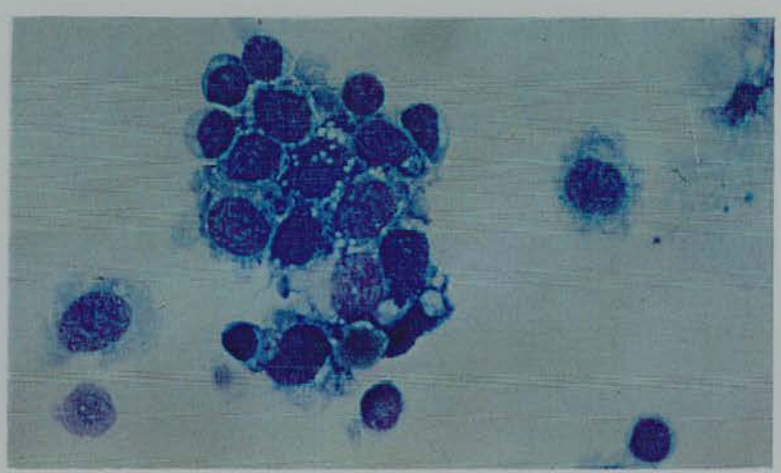


Prints 5 - 6: **Touch imprints and cytocentrifugation specimens of low grade B cell lymphomas studied**

L6 Touch

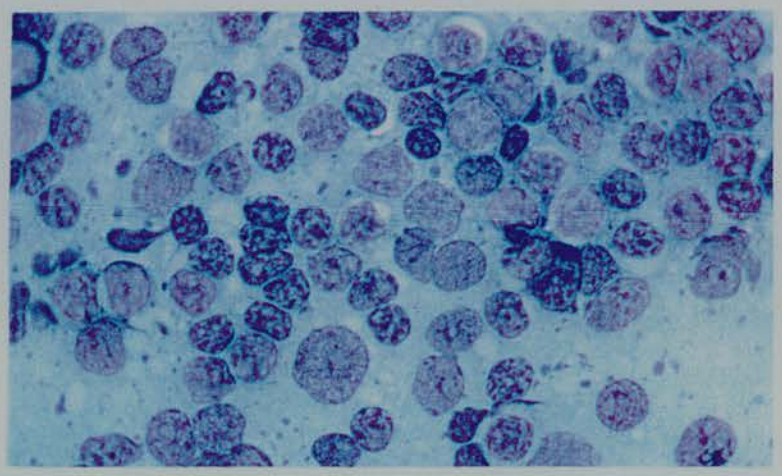


L6 Culture

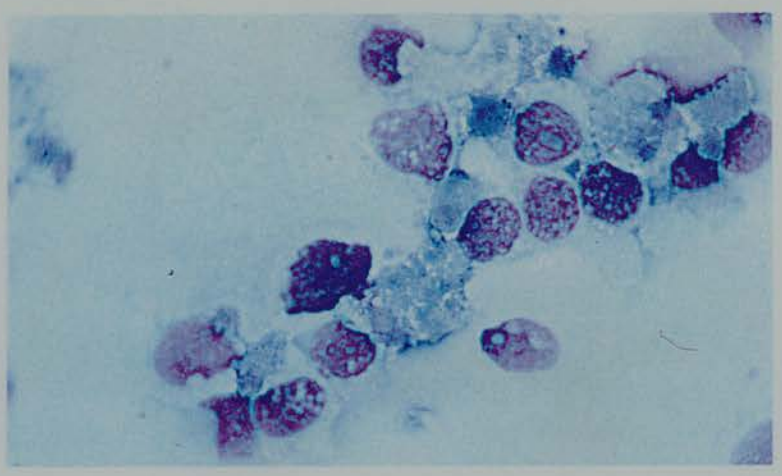


Prints 7 - 8: **Touch imprints and cytocentrifugation specimens of low grade B cell lymphomas studied**

L12 Touch



L12 Culture



LEGENDS TO PRINTS

Print 1: Touch preparation of original node biopsy, case L3, lymphocytic lymphoma. Lymphocytes with coarse, clumped chromatin are seen. MGG x 1000.

Print 2: Case L3 cytospun cultures at day 10. Lymphocytes with clumped chromatin are seen with scattered fibroblasts. Note the increase in cytoplasm and cytoplasmic vacuolation compared to Figure 5. MGG x 1000.

Print 3: Touch preparation of original node biopsy, case L5, follicular lymphoma. The tumour is mostly composed of centrocytes with occasional nucleolated centroblasts. MGG x 1000.

Print 4: Case L5 cytospun cultures at day 15. Centrocytes with vacuolated cytoplasm are seen tightly clustered around a mouse fibroblast. MGG x 1000.

Print 5: Touch preparation from original node biopsy, case L6, lymphoplasmacytoid lymphoma with a high blast content. Note the polymorphic mixture of large nucleolated blasts, lymphoplasmacytoid cells and intermediate sized lymphocytes with irregular shaped nuclei. MGG x 1000.

Print 6: L6 cytospun cultures at day 16. Hyperchromatic, polymorphic lymphocytes are seen adherent to the mouse fibroblasts. Note the preservation of the range of cytological types seen in Figure 1. MGG x 1000.

Print 7: Growth L12 - Touch . Follicle centre lymphoma with excess blasts (grade 3).

Print 8: L12 day 16 - cytospun culture - mostly centrocytes showing some degree of vacuolar with adherent fibroblasts.

5.4 T cell and monocyte growth in the IL3/IL10 anti-CD40 culture system (Tables 3 and 4)

The percentage of T cells and monocytes remained reasonably constant during the 23 day study period. In the 8 lymphoma specimens studied the mean percentage of T cells (CD3 positive) on day 1 was 33% (SD 24.6) by day 15 this was 35% and fell to 18% (SD 14.2) by day 23.

The wide range was maintained during culture with a gradual loss of T cells after 15 days. The number of monocytes (CD14 positive) was consistently low - mean 5% (SD 2.7) with a gradual increase to 9% (SD 2.6) by day 23. These were not analysed in the IL4/anti-CD40 system.

Table 3: Table showing CD3% Expression with time

Lymphoma	Histology	Day 1	Day 5	Day 10	Day 15	Day 23
L2	FCL	38	74	67	58	39
L3	SLL	12	11	6	22	11
L5	FCL	28	9	12	35	11
L6	SMZL/DLBCL	88	75	46	62	19
L9	SLL	14	24	13	12	9
L10	FCL	8	11	10	28	1
L11	FCL	26	25	30	23	12
L12	FCL	50	33	35	40	44
Tonsil		35	13	13	12	19

Table 4: Table showing CD14% Expression with time

Lymphoma	Histology	Day 1	Day 10	Day 23
L2	FCL	5	16	13
L3	SLL	2	2	6
L5	FCL	2	9	7
L6	SMZL/DLBCL	7	12	8
L9	SLL	4	3	12
L10	FCL	4	5	12
L11	FCL	11	5	7
L12	FCL	5	9	7
Tonsil		9	2	3

5.5 Growth fraction of cultured lymphomas (see Figure 1, p.92)

Results are grouped according to whether there was morphological evidence of blastic transformation (on the histology) or not. Transformation was seen in 1 follicle lymphoma and 1 splenic marginal zone B-cell lymphoma. Results were compared to those seen on day 10 in the same lymphomas grown in IL4/anti-CD40. 1 follicle centre lymphoma grade 1, underwent blastic transformation in culture (L2). This was seen on repeated culture attempts.

(a) *IL3/IL10/anti-CD40*

(i) No evidence of transformation - Growth Fraction (GF) on day 1 (mean of 6) was 7.5% (standard deviation, SD 4.9). This was stable for the first 10 days (mean 6.5%), (SD 5.1) rising to a mean of 14.1% thereafter (SD 5.8).

(ii) Transformed (mean of 2 cases) - These had a higher initial growth rate (mean 12.0%, SD 2.0 on day 1), falling to 7.2%, (SD 0.8) by day 10 and subsequently increasing to 22.5% by day 23 (SD 4.5).

(b) *IL4/anti-CD40*

(i) No evidence of transformation:- the GF on day 1 remained the same at 7.5% (SD 4.9) (mean of 6 cases) but by day 10 it had risen to 21.7% (SD 8.8).

(ii) Transformed:- the initial growth rate of 12.0% (SD 2.0) (mean of 2 cases) rose by day 10 to 28.3% (SD 4.8).

5.5.1 *Cell Number (see Figure 2, p.93)*

The cell count rose slowly using IL3/IL10/anti-CD40 with an approximate 2 fold expansion over 3 weeks in those showing no evidence of transformation compared with a 5 fold expansion in those showing transformation. In 4 cases on day 23 when the cells appeared to stop growing, IL4 was added to the culture system to stimulate further growth (L2, L6, L5, L11). This was not effective.

5.6 Establishing a Monoclonal Expansion (see Table 5)

2 colour staining revealed light chain restriction in all 8 cases prior to culture. In the IL3/IL10 anti-CD40 system, light chain restriction could be demonstrated in all at day 10. The corresponding results

in the IL4 T cell/IgD bearing cell depleted population showed a large degree of concordance. There was no evidence at this stage of any reduction in monoclonality. At day 23, it could be seen in 4. Expression of light chains was too poor to analyse in the other 4, and in 3 of these cases there was evidence of blastic transformation on cytocentrifugation specimens at this time.

Table 5: Table showing Light Chain Restriction at Day 10 in cultured samplescomparing growth in IL3/IL10 (no T cell depletion) versus that in IL4 (T cell and IgD bearing cells depleted)

Lymphoma	Histology	Day 1	IL3/IL10 Day 10	IL4 Day 10
L2	FCL	100%λ	93%λ	95%λ
L3	SLL	99%λ	99%λ	99%λ
L5	FCL	99%λ	82%λ	Poor Expression
L6	SMZL/DLBCL	96%λ	90%λ	Poor Expression
L9	SLL	99%λ	98%λ	98%λ
L10	FCL	97%λ	95%λ	78%λ
L11	FCL	97%λ	95%λ	75%λ
L12	FCL	90%λ	88%λ	61%λ
Tonsil		κ + λ	κ + λ	κ + λ

5.7 B Cell Phenotype during Cell Culture

(a) CD38 (see Figure 3, p.94 and Table 6)

3 groups were studied: a) follicle centre lymphoma, grown in IL4/anti-CD40 (2 samples); b) follicle centre lymphoma, grown in IL3/IL10/anti-CD40 (5 samples); c) non-follicle centre lymphomas grown in IL3/IL10/anti-CD40 (3 samples. Initial mean expression of CD38 in the follicle and non-

follicle centre groups was 46% (SD 10.7) and 30% (SD 17.6) respectively. Those grown in the IL3/IL10 system maintained CD38 expression at over 50% of original at day 5 and 20% of original at day 10 with an increase to over 35% by day 15. The lymphomas grown in IL4 lost almost all CD38 +ve cells within 5 days with no evidence of regrowth. The maintenance was not due to staining of T cells as these remained in all the groups and 2 colour staining CD3 PE/CD38 FITC failed to show more than 5% of cells coexpressing both.

Table 6: Table showing CD38 % expression with Time.

Lymphoma	Histology	Day 1	Day 5	Day 10	Day 15	Day 23
L2	FL	56	28	27	46	18
L3	SL	31	13	2	8	8
L5	FL	34	27	19	24	7
L6	LPC	51	35	21	29	17
L9	LPC	8	8	2	2	1
L10	FL	65	16	3	2	1
L11	FL	59	31	4	2	2
L12	FL	60	38	7	25	21

(b) CD19 (see Figure 4, p.95 and Tables 7a and 7b)

The follicle centre lymphomas were grown in IL3/IL10/anti-CD40 (5 lymphomas) or in IL4/anti-CD40 (2 lymphomas) and the non follicle centre lymphomas (3 samples) were only grown in IL3/IL10 anti-CD40. Mean expression was 35% (SD 14.1), 23% (SD 5.0) and 54% (SD 27.7) respectively. IL4 was the greatest promoter of CD19 cells

initially. The IL3/IL10 groups showed a small smaller variation compared to the original samples than the IL4. There was loss of CD19+ve cells in all cultures by day 23 - the levels being stable for the first 15 days (see Tables 7a and 7b).

Table 7a: Table showing CD19 % Expression with Time in IL3/IL10.

Lymphoma	Histology	Day 1	Day 5	Day 10	Day 15	Day 23
L2	FCL	34	24	30	34	24
L3	SLL	89	91	92	87	63
L5	FCL	18	60	46	61	20
L6	SMZL/DLBCL	21	16	42	88	13
L9	LPC	53	80	59	35	2
L10	FL	57	91	32	16	8
L11	FL	44	63	46	22	3
L12	FL	23	40	15	12	11
Tonsil		67	75	45	66	47

Table 7b: Table showing CD19 % Expression with Time in IL4.

Lymphoma	Histology	Day 1	Day 10	Day 24	Day 36
L2	FCL	27	68	60	3
L12	FCL	62	53	32	0

(c) CD10 (see Figure 5, p.96 and Tables 8a and 8b)

Only follicular lymphomas expressed this. 5 were grown in IL3/IL10/ anti-CD40; 2 in IL4/anti-CD40. Mean expression was 46% (SD 21.6) (IL3/IL10) and 18% (SD 14.0) in (IL4) compared to original expression. Both groups followed a

similar pattern involving first the loss of CD10 bearing cells followed by an increase in their number peaking on day 10 in the IL4 system and day 15 in IL3/IL10.

Table 8a: Table showing CD10 % Expression with Time.

Lymphoma	Histology	Day 1	Day 5	Day 10	Day 15	Day 23
L2	FCL	30	20	20	31	14
L3	SLL	1	2	2	10	8
L5	FCL	32	45	29	37	10
L6	SMZL/DLBCL	2	18	10	25	12
L9	SLL	2	7	8	7	16
L10	FCL	86	70	12	2	17
L11	FCL	51	41	12	16	28
L12	FCL	30	44	6	N/A	12
Tonsil		14	3	3	5	6

Table 8b: Table showing CD10 Expression in IL4

Lymphoma	Histology	Day 1	Day 10	Day 24	Day 36
L2	FCL	46	46	17	9
L12	FCL	30		6	4

(d) CD22 (see Figure 6, p.97 and Table 9)

This was analysed in 5 follicle centre lymphomas and 3 non follicle centre grown in IL3/IL10 only. Expression was initially 52% (SD 15.7) and 20% (SD 4.9) respectively. A gradual loss of expression was seen in the follicle centre lymphomas whilst the non follicle centre ones decreased expression gradually.

Table 9: Table showing CD22 % Expression with Time in IL3/IL10

Lymphoma	Histology	Day 1	Day 5	Day 10	Day 15	Day 23
L2	FCL	53	29	34	45	41
L3	SLL	19	19	14	14	8
L5	FCL	34	37	35	48	16
L6	SMZL/DLBCL	14	20	46	60	28
L9	SLL	26	43	21	18	9
L10	FCL	69	78	28	12	18
L11	FCL	69	52	46	36	19
L12	FCL	34	52	28	33	35
Tonsil		59	69	39	61	44

(e) CD34

No cells bearing this phenotype were detectable using flow cytometry.

5.8 *Bcl-2* Production (see Figure 7, p.98 and Table 10 for Individual Results)

Bcl-2 protein was measured in growing and resting cells (the cells were separated by PI binding) in IL3/IL10 and IL4. Percentages are quoted versus a negative control.

a) IL3/IL10

One lymphoma - (L3) did not express *bcl-2*, initially, however, after 10 days in culture 93% of cells expressed it. The follicle centre lymphomas expressed it quite strongly at first (mean 59% ± SD35) whilst the non-follicle centre ones expressed it to a lesser degree (mean 31% SD40).

Expression in the follicle centre group remained stable falling at the end of culture whilst in the non follicle centre group expression was upregulated. In both groups there was a tendency for resting cells in culture to express *bcl-2* more strongly than the growing cells during *in vitro* culture, at variance with the initial finding.

b) IL4

Expression was assessed at day 10. Overall, the tumours had a high level of expression, however, the small lymphocytic lymphoma failed to upregulate *bcl-2* in this system to the same degree as in IL3 + IL10 suggesting that the choice of cytokine might have different effects on different lymphomas.

Table 10: Table showing *bcl-2* expression with time in growing and resting cells

Lymphoma	Histology	Day 1		Day 10 IL3/IL10		Day 10-14 IL4	
		Growing	Resting	Growing	Resting	Growing	Resting
L2	FL	100	91	36	38	79	90
L3	SL	0	0	93	93	9	28
L5	FL	11	8	20	53	27	33
L6	LPC	5	14	9	16	30	59
L9	LPC	92	86	67	97	100	100
L10	FL	59	25	82	79	100	100
L11	FL	30	21	63	47	100	100
L12	FL	97	91	71	71	10	20

5.9 Discussion

The IL3/10 anti-CD40 stromal cell culture system provides an improved model for studying low grade lymphoma growth. Unlike its predecessors it produces a low growth fraction throughout the period studied. It supports follicle centre and non follicle centre B cell lymphomas equally well and morphology correlates well with the original lymphoma. The demonstration that no prior T cell depletion is required is a useful finding in that it simplifies the culture system. It may be that various T cell derived cytokines and adhesion molecules are critical in the maintenance of B cell morphology.

The ability to freeze and then resuspend the lymphoma cell suspensions without substantial loss of viability greatly enhances the flexibility of the system and allows direct comparison of lymphomas taken at various stages of their history. Proof of monoclonal expansion comes from light chain restriction studies. These confirmed that despite the fact that there was no T or IgD bearing cell depletion, there was no suggestion that the cells in this system were less likely to show restriction than those in IL4. Cytogenetic analysis was not undertaken in part because of the slow growth rate made colcemid treatment difficult to time and also because of the limited number of cells - particularly in the first 5-10 days. The morphological variations between the tumours - and the retention of parent tumour morphology seen on cytocentrifugation specimens is further evidence that what was growing did not represent a benign reactive B cell population as this would be expected to look similar in all cases studied.

The model appears to support 3 phases of cell growth and development as assessed by FACS analysis. During the first 10 days, there is a gradual loss of mature (CD38+ve) cells and a relatively low growth fraction. The total number of cells may actually fall during this time (see Figure 9). For the next 5-7 days, as cells which are proliferating start to mature, the percentage of more mature cells increases again. In the last stage, although cells are still dividing it appears that they fail to mature beyond 23-25 days. This may represent a failure to recruit newly divided cells into the maturation pathway.

In 4 lymphomas at day 23, the addition of IL4 failed to induce further proliferation or maturation and there is no suggestion of a CD34+ve cell pool which replenishes the system. The length of time this culture system can continue for is less than that described for IL4/anti-CD40[34], as the cells do not respond to IL4 at this stage, whilst when all 3 cytokines are combined initially (data not shown) there is no evidence of antagonism (although CD38+ve cells are rapidly lost). It must therefore be concluded that at least part of the IL4 sensitive sub-population is not supported by IL3/IL10. Unfortunately although IL4 driven B lymphoma cells will proliferate for longer this is at the expense of loss of B cell phenotype as judged by CD19 and CD22 expression.

The role of the stromal cells in such a culture system is complex. Neither IL3 alone, nor IL3 in combination with soluble anti-CD40,

lead to B cell proliferation. Whilst similar concentrations combined with stromal cells do cause B cells to divide[71] indeed short term culture of follicular lymphoma cells *in vitro* appears to be inhibited by IL3 - at least for 72 hours[45]. The stromal cells were stained with anti follicular dendritic cell antibody (DRC-1) to ascertain if they fulfilled this role, however, they were negative for this as well as the adhesion molecules CD11a and CD54. They stained weakly for CD58 suggesting that these adhesion molecules could not provide the necessary B cell ligands which the normal stromal cells did.

Bcl-2 expression was maintained until the end of the time period studied when its reduction correlated with the increasing number of vacuolated cells which were presumably losing viability. Initially expression was higher in growing than resting cells but with time this was reversed. Cytokines have been shown directly to influence *bcl-2* expression as does cross linking of CD40. IL4 suppresses apoptosis in B-CLL by maintaining *bcl-2* levels[50], anti-CD40 does the same[30] and in germinal centre cells increases *bcl-2* expression. As constitutive *bcl-2* expression is not sufficient to prevent apoptosis presumably anti-CD40 works through other mechanisms as well. In particular, many growing cells did not express *bcl-2* in culture.

It is interesting that the non follicle centre lymphomas originally expressed *bcl-2* to a lesser extent than the follicle centre lymphomas, but by day 10 expression was equivalent. This was most marked in the small lymphocytic lymphoma which did not

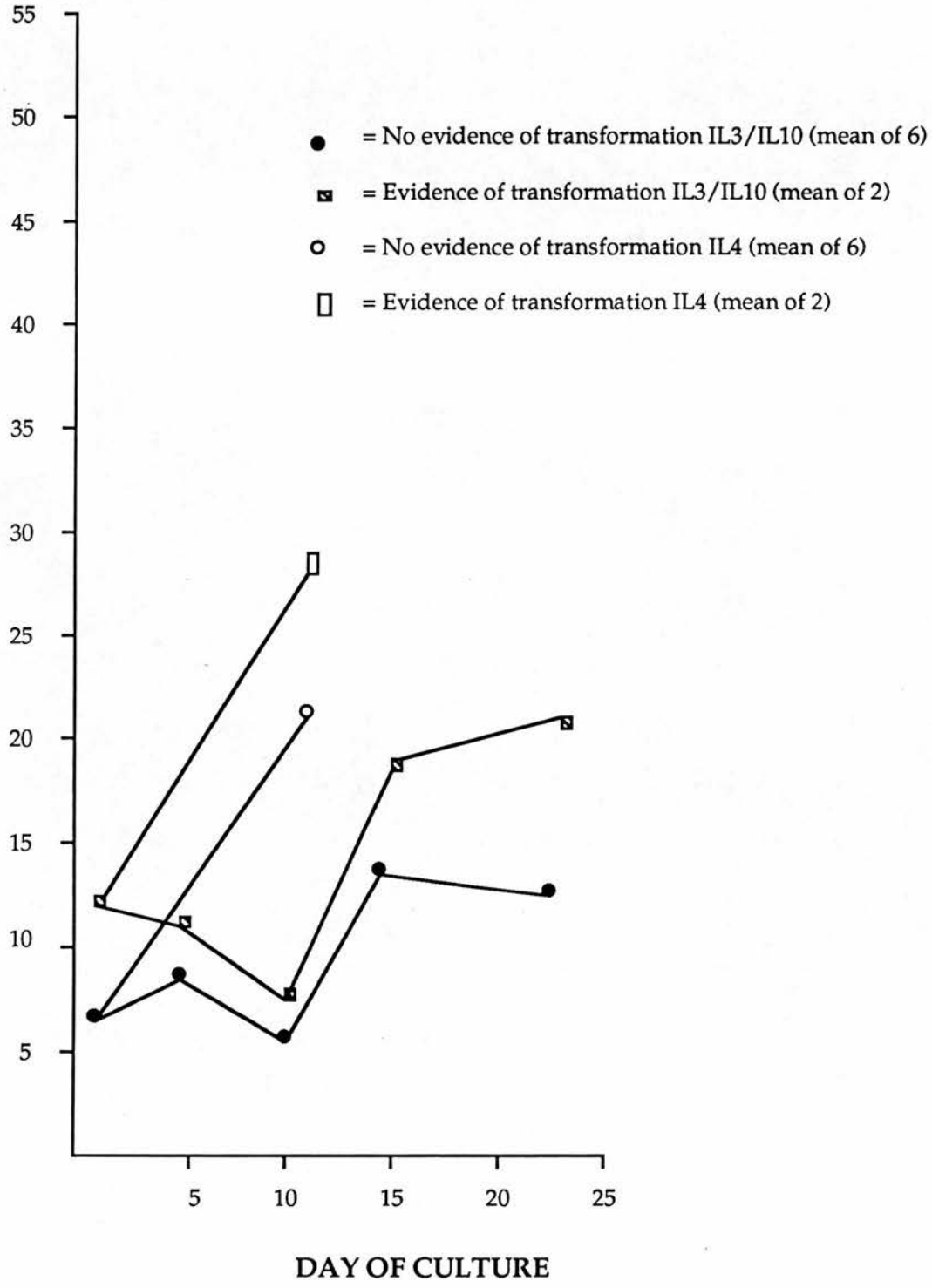
express *bcl-2* at all initially; presumably the IL3/IL10 and anti-CD40 was affecting expansion directly regardless of initial levels.

The prolongation of the presence of germinal centre cell equivalents (CD19+ve, CD38+ve) although much greater in IL3/IL10 as opposed to IL4 alone was also seen in cells cultured in IL3/anti-CD40. The cells strongly expressed *bcl-2* which suggests that IL10 may not be critical to their survival[58], that IL4 was unable to do this despite *bcl-2* upregulation (data not shown) again brings the central role of *bcl-2* into doubt.

Recently, it has been shown that *c-myc* which normally induces proliferation can induce apoptosis in serum deprived fibroblasts in the presence of various cytokines. Such an effect can be nullified[72] in the absence of growth promotion. Furthermore, it appears that the presence of p53 may be required to convert the proliferative signal into an apoptotic one[73]. In a similar way, it may be that the balance between *bcl-2* and its partner *bax* which induces apoptosis[74], is modulated by the cytokines IL3 and/or IL10 and/or anti-CD40 so that the absolute level of *bcl-2* may become irrelevant. Further investigation into the balance between *bax* and its candidate partners may be helpful in this system.

In summary, a modified culture system for low grade B cell lymphoma is described which maintains many of the *in vivo* morphological and growth characteristics for 20-25 days and may permit the *in vitro* investigation of drug sensitivity and mechanisms of drug resistance as well as the assessment of biological therapy.

Figure 1: Growth Fraction of Lymphomas with time



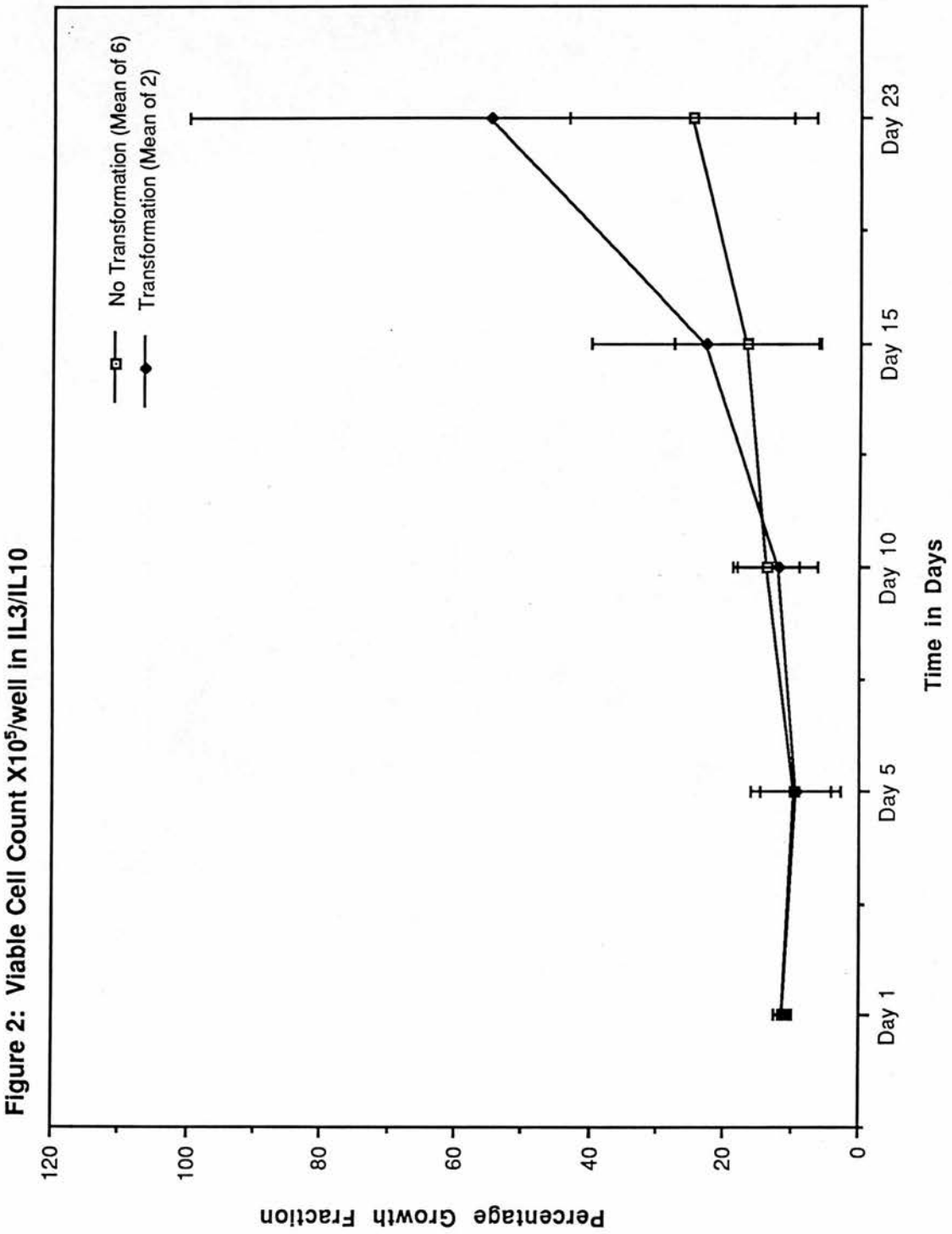


Figure 2: Viable Cell Count X10^5/well in IL3/IL10
No Transformation (Mean of 6)
Transformation (Mean of 2)
Percentage Growth Fraction
120
100
80
60
40
20
0
Day 1
Day 5
Day 10
Day 15
Day 23
Time in Days

Figure 3: CD38 expression as a percentage of original in low grade lymphoma in IL3/IL10 stromal cell system

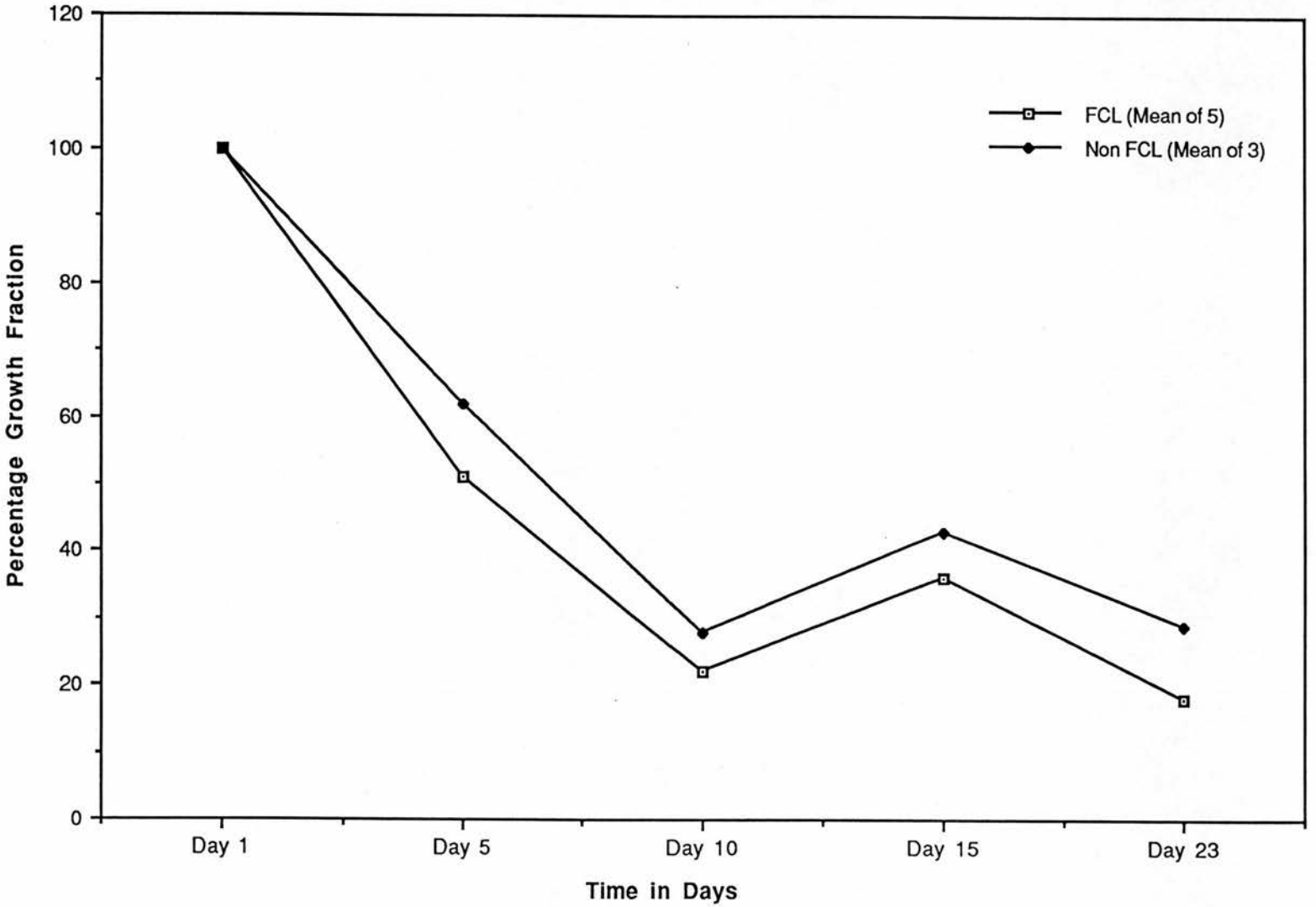


Figure 4: CD19 expression as a percentage of original in low grade lymphoma in IL3/IL10 stromal cell system

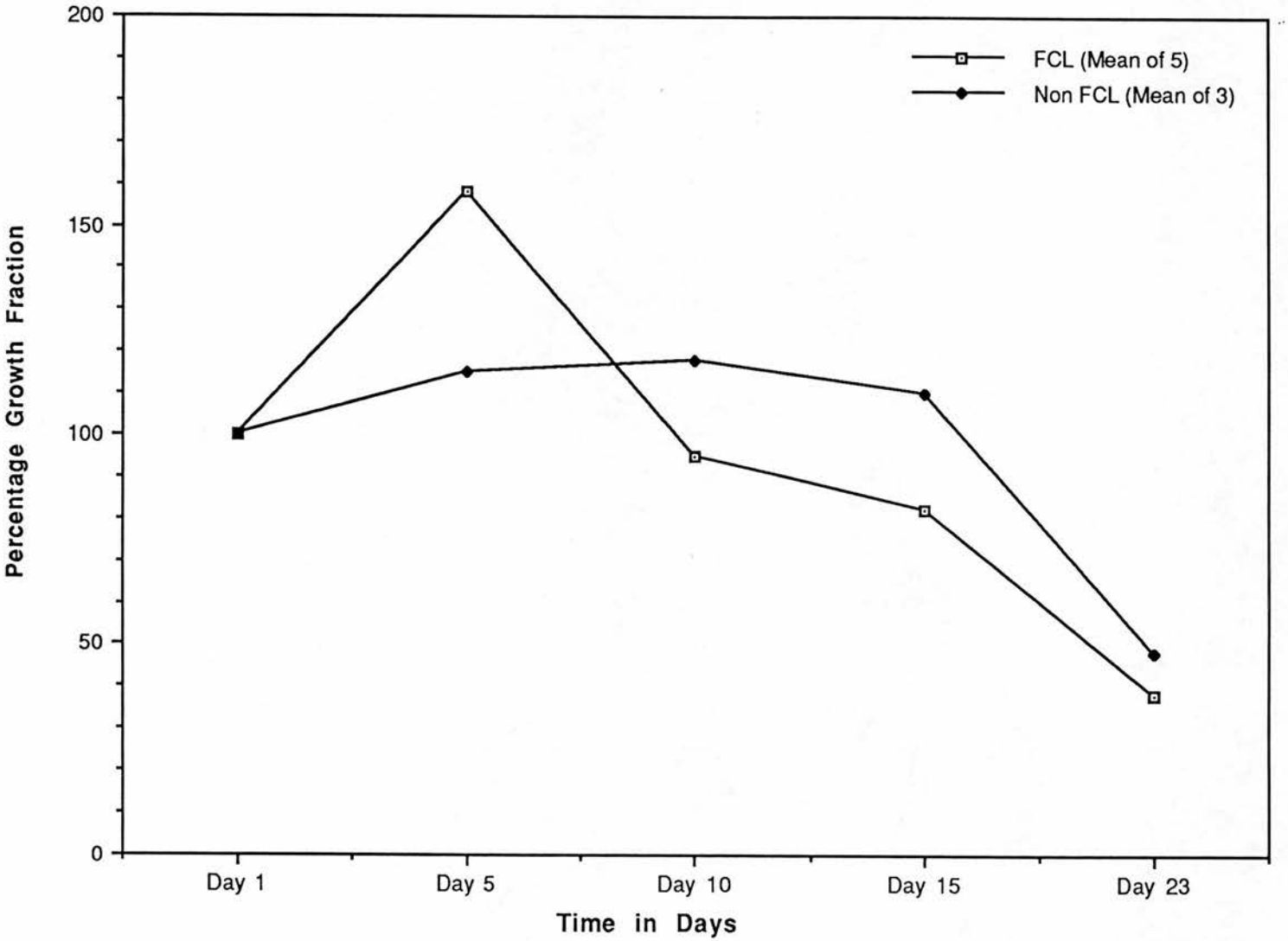


Figure 5: CD10 expression as a percentage of original in follicle centre cell lymphoma in IL3/IL10 stromal cell system

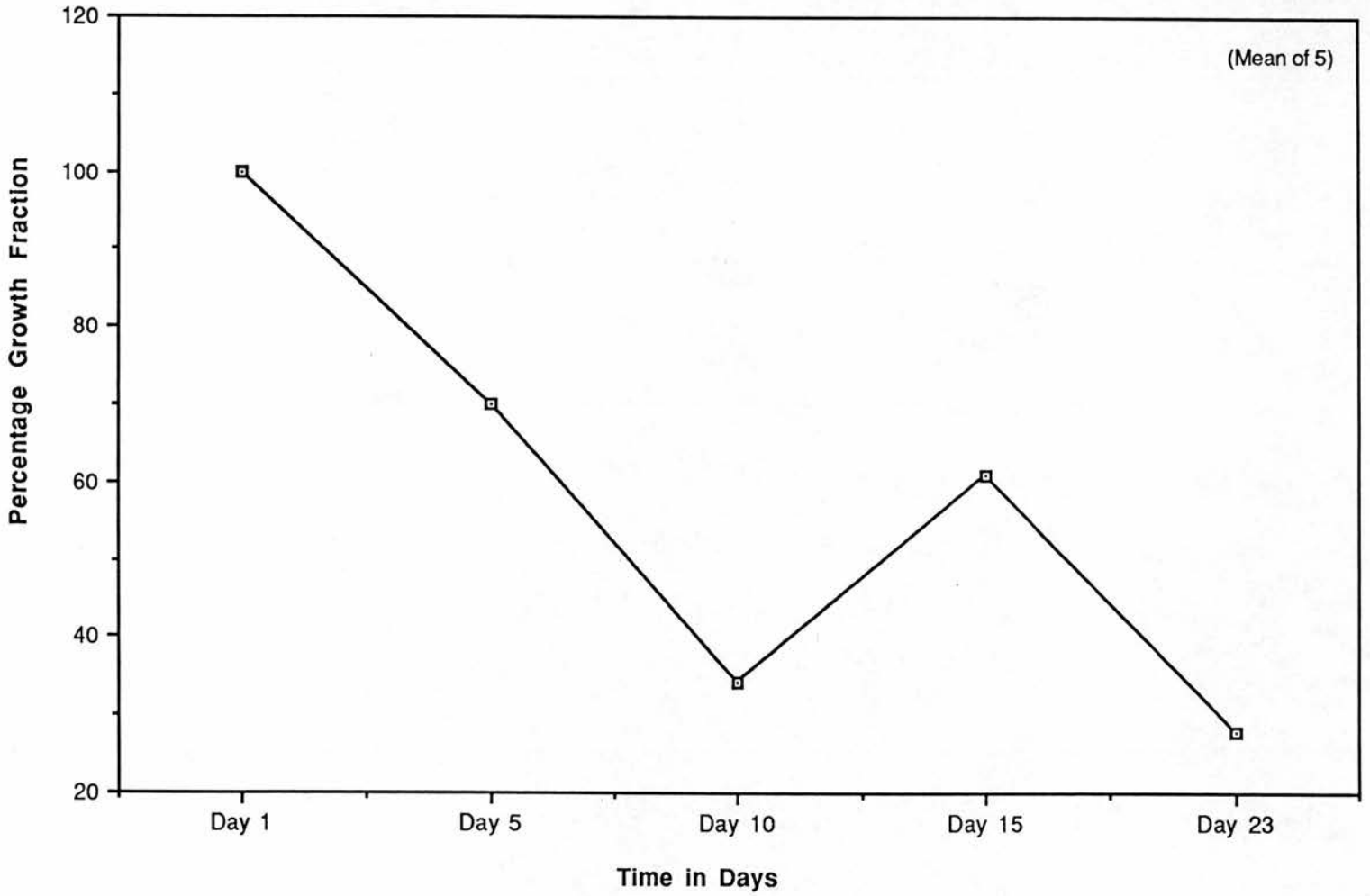


Figure 6: CD22 as a percentage of original in low grade lymphoma in IL3/IL10 stromal cell system

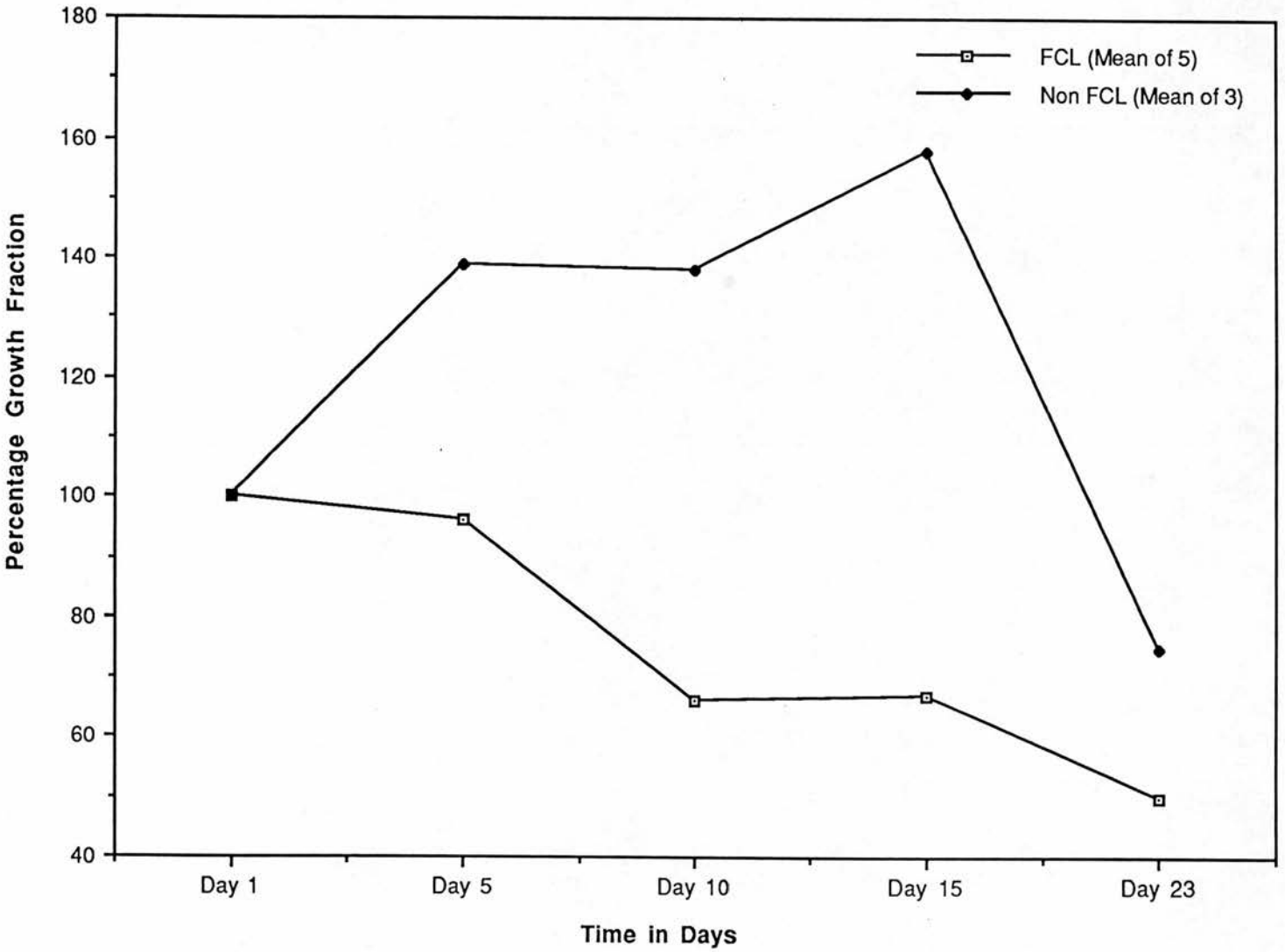
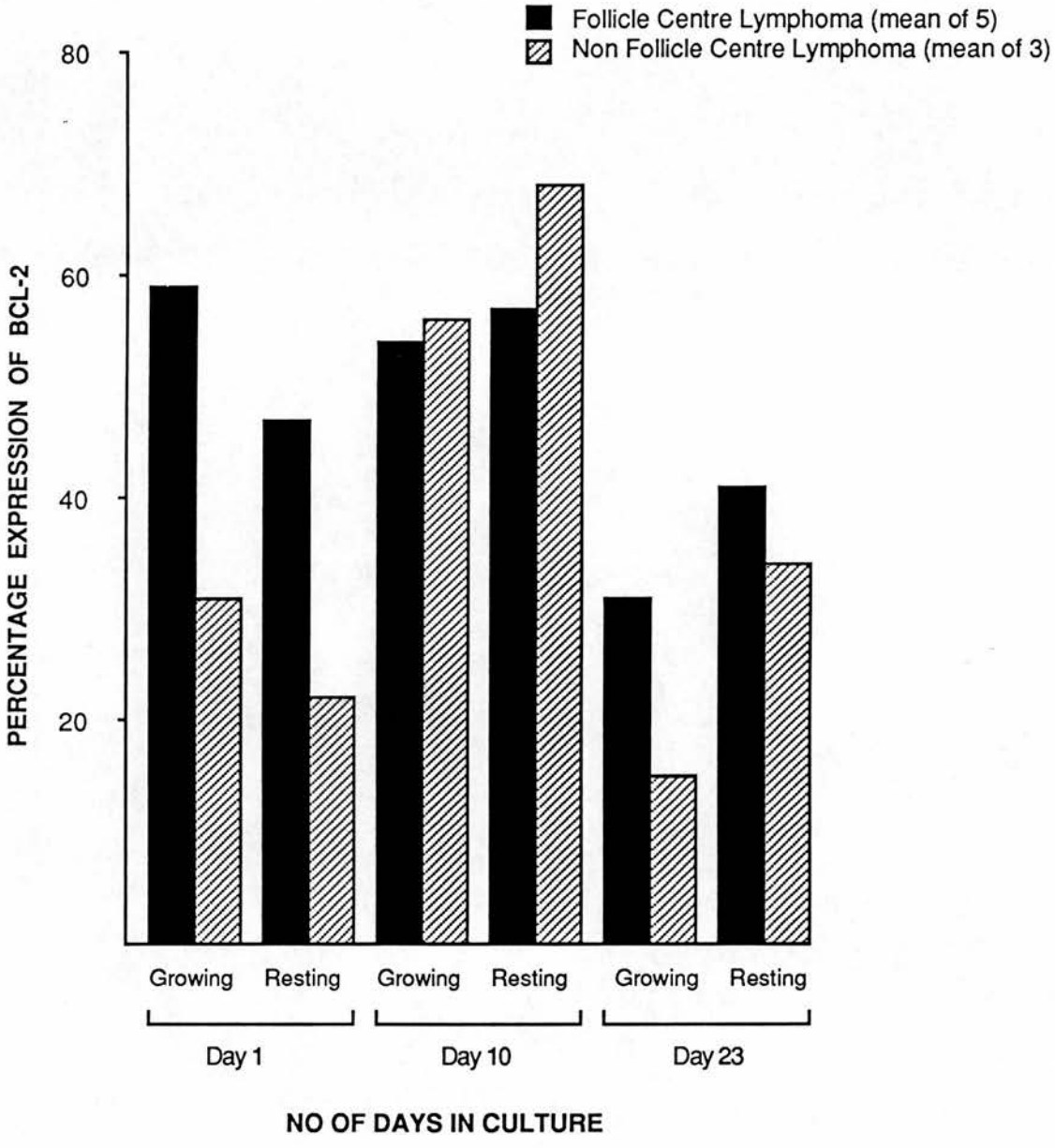


FIGURE 7

ANALYSIS OF BCL-2 PRODUCTION ACCORDING TO HISTOLOGY



CHAPTER 6

CULTURING HODGKIN'S LYMPHOMA

6. CULTURING HODGKIN'S LYMPHOMA

6. CULTURING HODGKIN'S LYMPHOMA

6.1 Introduction

The difficulty in studying this disease has required several methods of investigation to confirm the growth of the putative malignant cells *in vitro*. This chapter describes the use of these methods to establish the nature of the cells growing in culture.

6.2 Rationale for the IL3/IL10 anti-CD40 stromal cell system

Hodgkin's lymphoma has proved very difficult to study *in-vitro*. The putative malignant cell, the Reed-Sternberg-Hodgkin cell (RS-H) has proved resistant to *in-vitro* expansion and makes up a small proportion of cells within the affected lymph node[75,76]. The origin of the cell is unknown but a B or T cell origin is probable[70]. T and B cell markers are variably expressed although most LP-HL express CD20[70] (a B cell marker) and κ or λ light chain mRNA has been found in 50% of LP-HL suggesting a B cell origin for this subtype at least. EBV has long been linked to HL and 50% of cases express EBV latent membrane protein[70]. Its ability to induce the proliferation of infected B lymphocytes appears to require the production of hIL10[55]. RS-H cells have been shown to express CD40, but binding to its natural ligand (CD40L) mitotic activity did not occur in HL derived cell lines[37]. However, LMP-1 may upregulate CD40 in RS-H cells and this may represent a mechanism for EBV induced RS-H growth[77].

As there was now evidence that the IL3/IL10 anti-CD40 stromal cell system was able to maintain morphological and immunophenotypical features of the various low grade B cell lymphomas, the clear B cell characteristics of LP-HL, the possession of CD40 by RS-H cells, and the link between the EBV infection and hIL10 synthesis made the system attractive to study in HL.

Lymph nodes from patients with HL were taken, T cell depletion was carried out if the CD3 proportion exceeded 60%, and the remaining cells were cultured in the stromal cell system. Phenotyping for CD15 and CD30, DNA profiles using FACS analysis, light microscopy features on cytocentrifuged specimens were carried out. Clonality of the cultured cells was sought using conventional cytogenetic karyotyping in four cases as well as PCR analysis for IgH rearrangements on cells at various times in culture. Where numbers allowed CD30 expression based cell sorts were carried out using flow cytometry to compare the CD30-ve and CD30+ve populations.

6.3 Lymphoma characteristics at biopsy

The histology, EBV status and T cell portions as assessed by CD3 staining using flow cytometry is shown in Table 1 and the clinical status of the patients with regards to stage and number of previous relapses is shown in Table 2. $^{2}/_{6}$ cases were EBV positive and $^{3}/_{6}$ required T cell depletion. 3 patients were previously untreated.

Table 1

Lymphoma	Histology	EBV Status	CD3%	T Cell Depletion
L17	LP	-ve	78	Yes
L18	MC	-ve	78	Yes
L20	LP	-ve	36	No
L25	MC	+ve	82	Yes
L27	NS	-ve	40	No
L28	NS	+ve	85	No

L17, 18, 25 and 28 required T cell depletion prior to culture.

Table 2

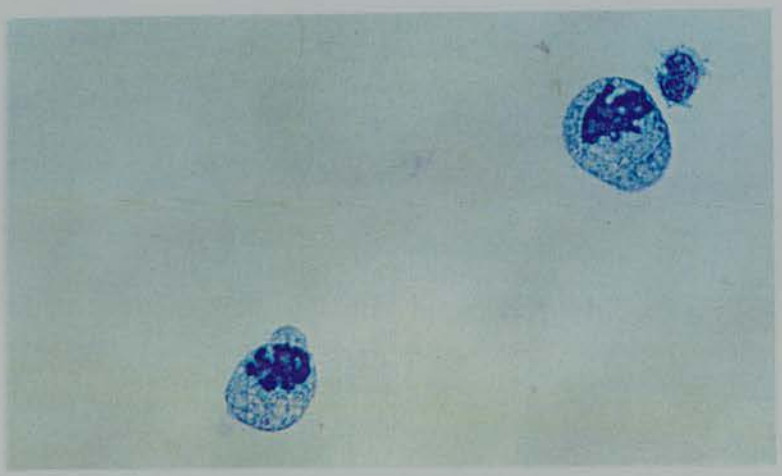
Lymphoma	Remission/Relapse	Stage
L17	Presentation	IIa
L18	Presentation	IIb
L20	1st Relapse	IIIb (Restaged)
L25	Presentation	IIa
L27	2nd Relapse	IIIax (Restaged)
L28	6th Relapse	IVa (Restaged)

6.4 Light Microscopy of Cytocentrifugation Specimens (see Table 3)

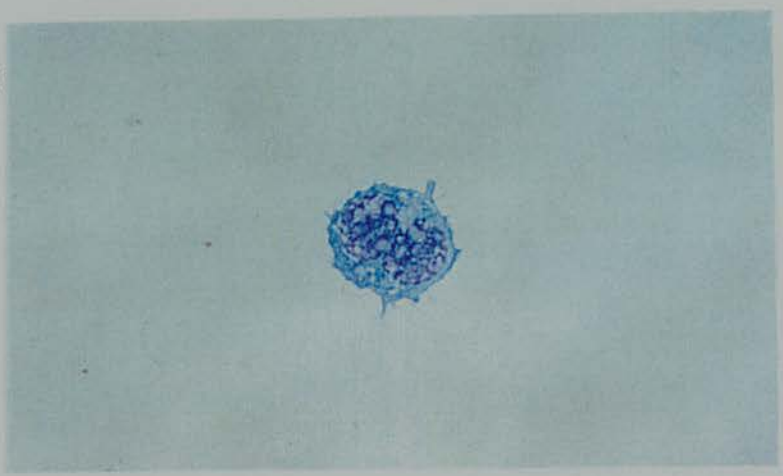
The cases were studied at 3 points during culture. Photographs of selected cases (prints 1-5) with the benign control given for comparison. In 3 cases cell sorting allowed direct visualisation of the CD30 positive and negative cells. The CD30 negative sorts generally contained fibroblasts and vacuolated lymphoid blasts, whilst the CD30 positive sorts contained the bi and multinucleated cells.

Prints 1 - 3: Cytocentrifugation specimens of Hodgkin's lymphoma cultures

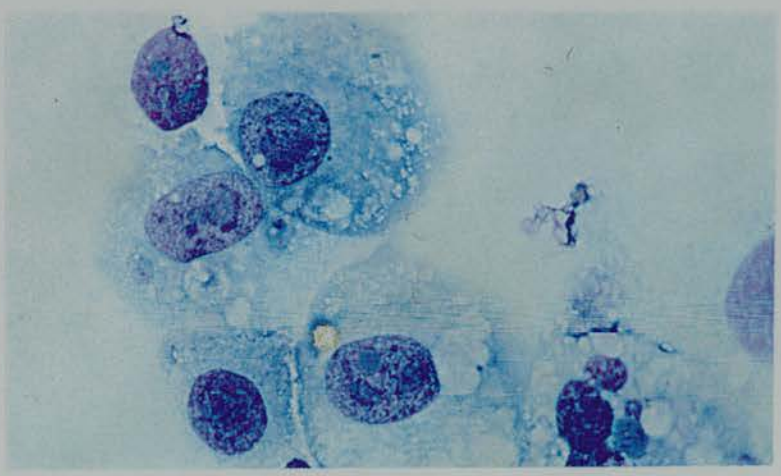
L25, CD30+ve, 1



L25, CD30+ve, 2

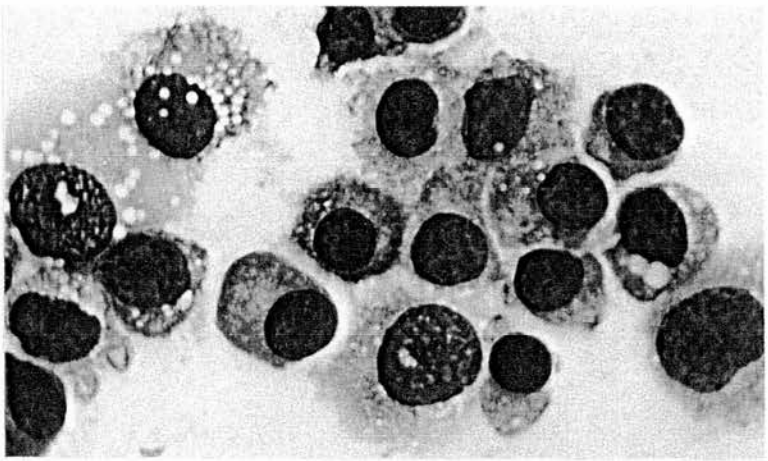


L25, CD30-ve

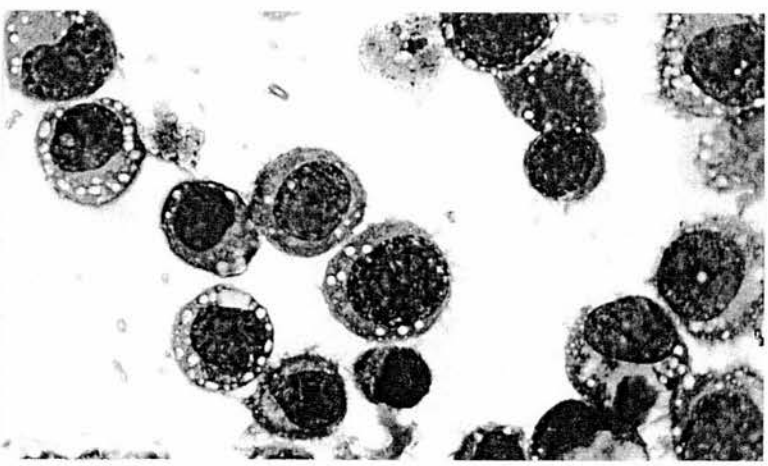


Prints 4 - 5: **Cytocentrifugation specimens of Hodgkin's lymphoma cultures**

L18, Day 9



L20, Day 15



LEGEND FOR PRINTS - HODGKIN'S LYMPHOMA

Print 1: L25 - Day 14

CD30+ve cell sort - showing mitotic cells.

Print 2: L25 - Day 14

CD30+ve cell sort - classical Reed-Sternberg cell.

Print 3: L25 - Day 14

CD30-ve cell sort - showing mouse fibroblasts and small mononuclear cells.

Print 4: L18 - Day 15

Mononuclear blast cells with occasional multinucleated cells. Some cells show plasma cytoid features.

Print 5: L20 - Day 9

Large mononuclear cells interspaced with small lymphocytes. Some multinucleated and bilobar cells.

Table 3: Description of Cytocentrifugation Specimens

TUMOUR (HISTOLOGICAL SUBTYPE)	DAYS 5 - 9	DAYS 10 - 19	DAY 20+
L17 (LP)	Large numbers of blasts with multiple nucleoi and some nuclear pleomorphism.	CD30+ sort Large numbers of blasts as before with increased cytoplasmic volume and mitotic figures. CD30-ve sort Fibroblasts and vacuolated blast cells.	Blast cells as before and cell debris.
L20 (LP)	Large numbers of mononuclear blast cells with plentiful cytoplasm. Some multinucleated and multilobated forms.	CD30+ve cell sort Large numbers of blast like cells as on day 9. CD30-ve cell sort Fibroblasts and cell debris. Heavily vacuolated blasts.	Large numbers of blasts as on day 9. Some showing more cytoplasmic vacuolation.
L18 (MC)	Plentiful mononuclear blasts with abundant vacuolated cytoplasm. Clumpy growth. Rare binucleated cells.	Large numbers of mononuclear blasts and scattered multinucleated cells, some showing plasmacytoid features.	Plasmacytoid blasts and cell debris.
L25 (MC)	Scattered small cells of indeterminate type.	CD30+ve cell sort Plentiful blasts. Many cells in mitosis and frequent binucleated cells. (Print 3) CD30-ve cell sort Fibroblasts and cell debris. Some mononuclear blasts.	
L27 (NS)		Scattered small lymphocytes. Rare larger nucleolated blast cells.	
L28 (NS)	Many fibroblasts. Small cells of indeterminate type.	Rare large mononuclear blasts, small lymphocytes and some centrocyte like cells.	Rare mononuclear blasts with large nucleoli and plentiful cytoplasm.

6.5 CD15 and CD30 expression during culture - by FACS analysis (see Table 4 and Figures 1 - 3)

Upregulation of CD15 and CD30 were seen in all lymphoma cases although often the values were not identical. Overall, CD30 seemed to be more strongly expressed than CD15. In the reactive control (R1) no such upregulation occurred. In most cases this peaked at between day 10-19.

Table 4

Lymphoma	Day 1		Day 5-9		Day 10-19		>Day 20	
	CD15	CD30	CD15	CD30	CD15	CD15	CD15	CD30
L17	18	14	44	33	46	41	66	92
L18	9	1	18	24	33	57	19	26
L20	11	4	14	18	50	83	38	20
L25	6	3	-	-	63	76	-	-
L27	6	4	-	-	20	25	-	-
L28	15	33	33	35	54	50	85	72

Figure 1: FACS analysis showing histograms of CD15 and CD30 expression in Hodgkin's lymphoma pre and post culture

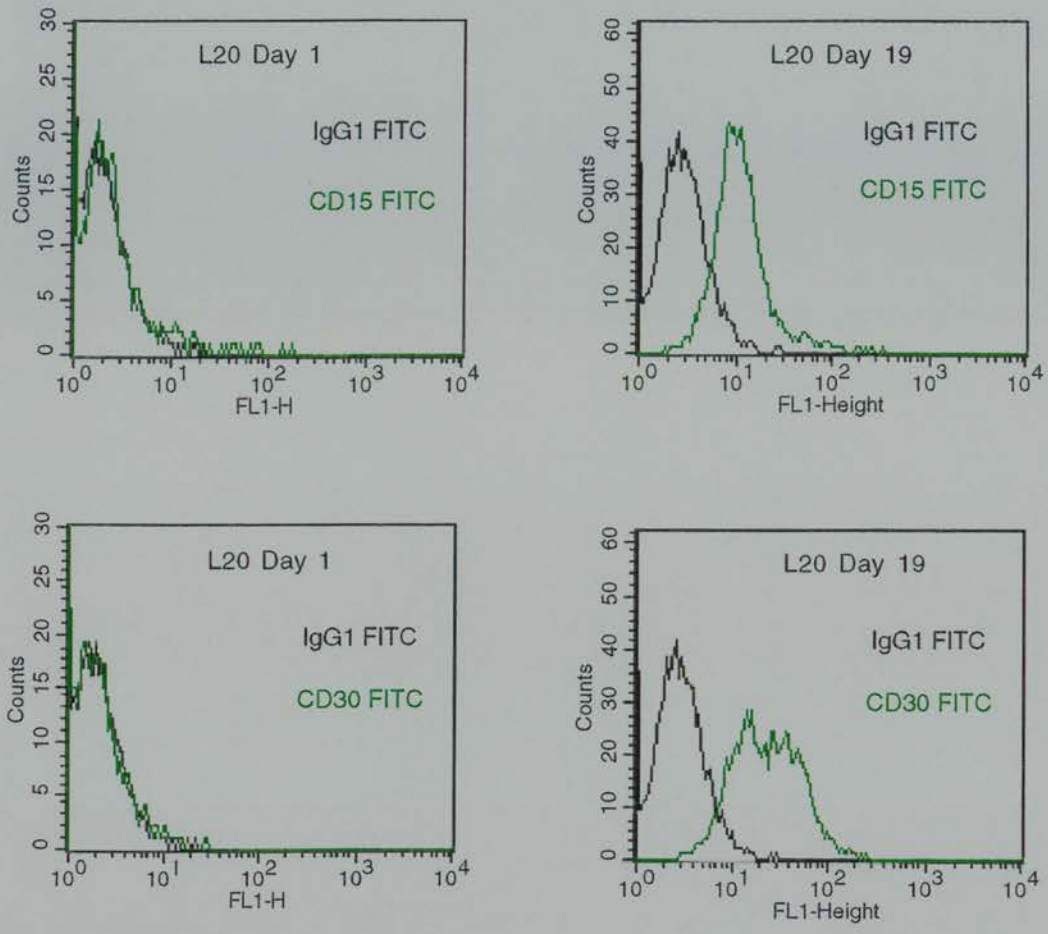


Figure 2: FACS analysis showing histograms of CD15 and CD30 expression in Hodgkin's lymphoma pre and post culture

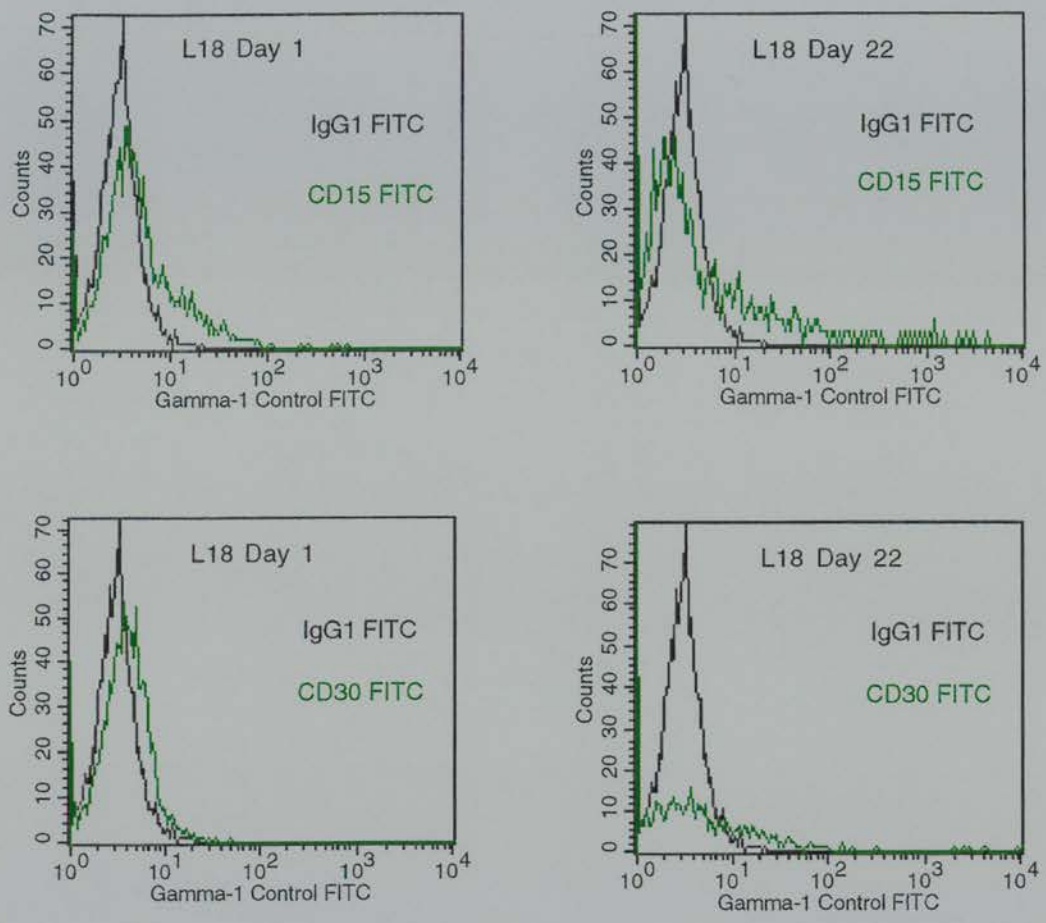
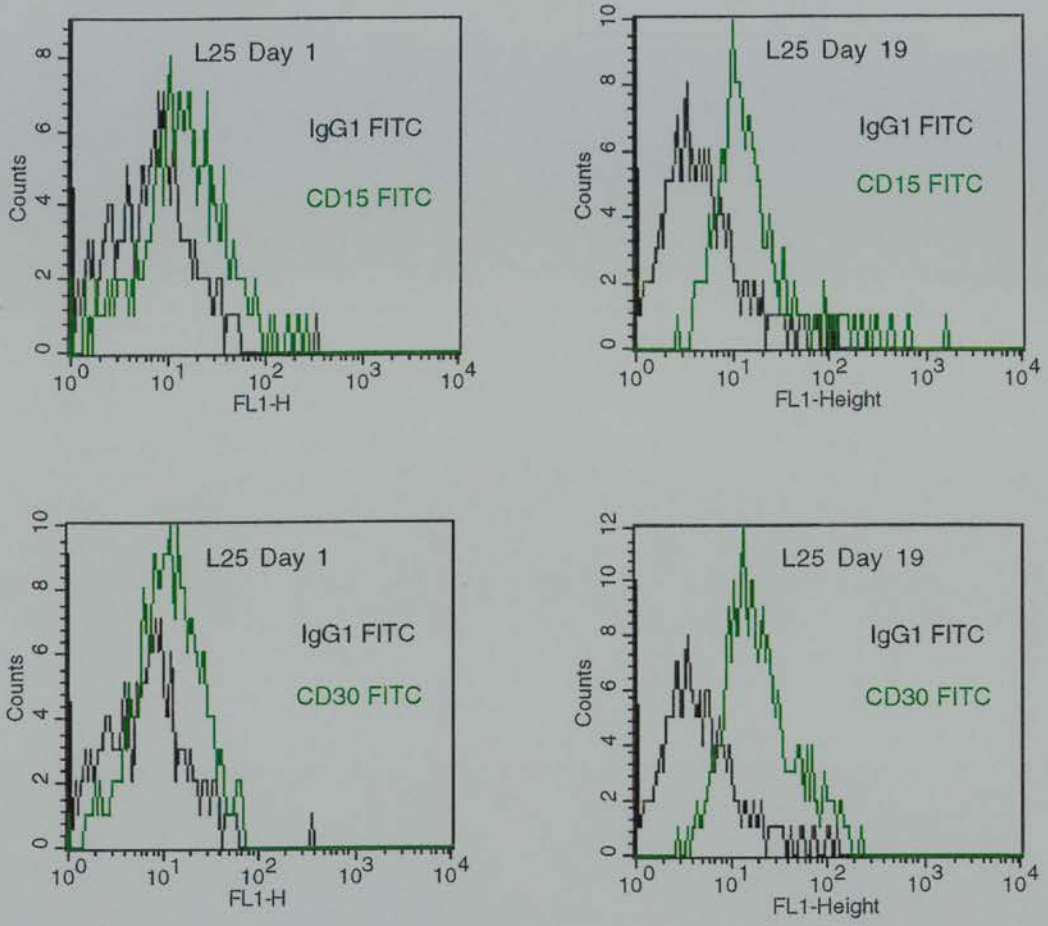


Figure 3: FACS analysis showing histograms of CD15 and CD30 expression in Hodgkin's lymphoma pre and post culture



6.6.1 *Light Chain Expression*

Only L17 (κ) and L20 (λ) (LP-HL) expressed light chains. In both cases using 2-colour FACS analysis, the original light chain restricted persisted in culture.

6.6.2 *CD19 and CD22 Expression*

Both the lymphocyte predominant tumours expressed B cell markers CD19 and CD22 by FACS. These were maintained during culture. In other tumours (L18, 25 and 28) CD19 expression remained at a low level and may have represented benign B cells.

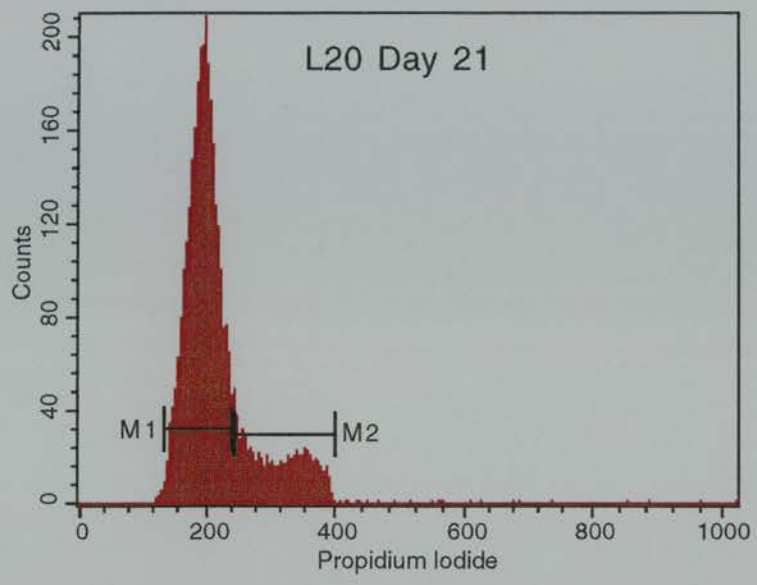
6.7 Growth Fraction in vitro (see Tables 5 and 6 and Figures 4 and 5)

This was measured using either PI or Hoechst uptake. In L17, L20 and L25 sufficient number of cells were available to allow sorting into CD30+ve and CD30-ve populations. When separated into positive and negative cells; the +ve population always seen have the higher growth fraction. The percentage represented the cells that were not diploid.

Table 5

Growth Fraction	Day 1	Day 5-9	Day 10-19	>Day 20
L17	6	19	13	18
L18	-	20	15	1
L20	9	35	15	14
L25	-	-	38	-
L27	-	-	26	-
L28	12	36	37	-
Reactive Control	13	38	23	25

Figures 4 - 5: FACS analysis of two cases of Hodgkin's lymphoma showing Propidium Iodide Histograms



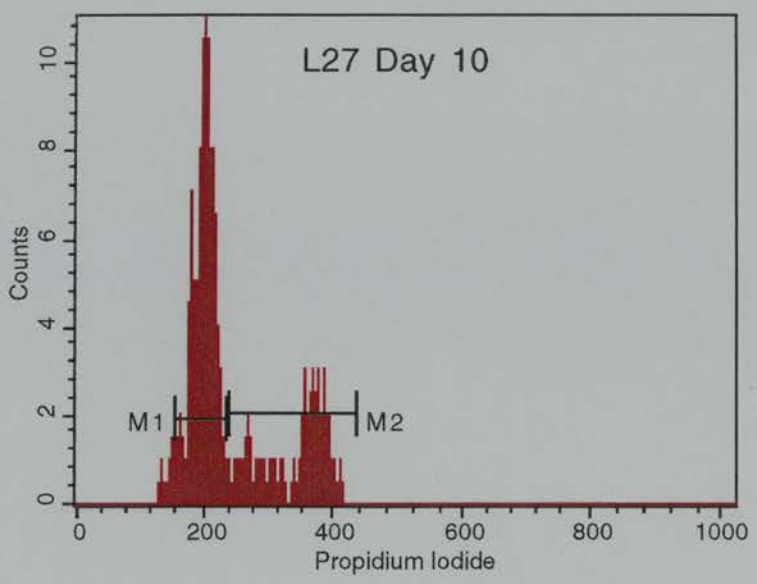


Table 6

Sorted Cells - Cells sorted on Day 15						
	CD30-ve			CD30+ve		
	M1	M2	M3	M1	M2	M3
L17	69	5	1	69	10	2
L20	81	4	0	61	11	1
L25	46	11	2	57	15	1

M1 - Diploid

M2 - Tetraploid

M3 - Hypertetraploid

6.8 Cytogenetics (see Table 6)

4 out of the 6 samples were evaluated by conventional karyotyping. Although abnormalities were seen in culture, no clonal changes were seen and no consistent abnormalities could be found. Tetraploid cells were seen in cases L17, L18 and L25.

Table 7

RESULTS OF KARYOTYPING	
TUMOUR	**FINDINGS**
L17 (LP)	31 Cells Analysed Tetraploid Cell 29 46,XY 1 45,XY,-20
L18 (MC)	32 Cells Analysed 4 Hyperdiploid Cells - Probably Tetraploid 1 46,XX,del(1)(q?q?) 27 - Normal Karyotype
L20 (LP)	30 Cells Fully Analysed 15 Further Cells Checked for del(20)(q) $^{1}/_{45}$ del(20)(q) $^{29}/_{30}$ - Normal Karyotype
L25 (MC)	10 Cells Analysed Normal Karyotype Tetraploid Cells Seen

6.9 Molecular Analysis

Samples were considered to be +ve when a single band was detected on electrophoresis - see Table 8.

Table 8: Results of the PCR analysis for the detection of clonal IgH Rearrangement

L17	L18	L20	L25	L28	Benign Control	Fibroblasts Alone
Day 1 (Prior to Culture)		**+ve**	**+ve**	**+ve**	**-ve**	**-ve**
D8 (+)	D8 (+)				Day 9 -ve	
	D15 (+)	D9 (+)				
Day 14 CD30+ (+) Cell Sort			Day 14 CD30+ (+) Cell Sort			
Day 14 CD30- (-) Cell Sort			Day 14 CD30- (-) Cell Sort			
		Day 14 CD30+ (+) Cell Sort				
		CD30- (+) Cell Sort				
D22 (+)	D22 (-)		D19 (+)	D16 (-)	Day 16 -ve	
		D22 (-)				

6.10 Discussion

The primary culture of HL from cells obtained at biopsy has been demonstrated using the IL3/IL10 anti-CD40 stromal cell system. Compared to the pattern seen with low grade B cell NHL, the growth fraction seemed to be much higher than the *in-vitro* rate as studied using PI. The fact that the CD30+ve population had a

higher growth fraction than the CD30-ve population suggests these cells to be actively dividing rather than CD30 being acquired as a maturation effect. The cytokines and stroma presumably antagonised the effect of CD40 ligation to down regulate CD30 expression[37]. In the reactive controls, little upregulation of CD15 and CD30 occurred, discounting the suggestion that the major upregulation might just be a culture artefact. However, it was consistent with the findings of Stein et al[77] that some CD30 upregulation follows activation of lymphocytes.

Unfortunately, PI and Hoechst 3342 cannot differentiate between resting and tetraploid cells and dividing diploid ones. However, the presence of small numbers of cells in the M3 band of hypertetraploid cells suggested that the hyperdiploid cells might be dividing as well. It was of note that the growth kinetics and expression of CD15 and CD30 appeared similar in the LP - and the non LP subtypes and it was only the lack of obvious light chain restriction which separated those subtypes. This represents supporting evidence that these diseases are correctly grouped together[6], and that LP-HC may simply have a more committed B-cell differentiation.

The lack of clonal cytogenetic abnormalities was seen in the sorted (CD30+ve) and non sorted specimens on repeated culture attempts. No consistent cytogenetic abnormalities have been found in HL[70] and the presence of Ig rearrangements could be too subtle to be recognised by conventional karyotyping techniques.

Occasional tetraploid cells were seen, although the quality of these was often too poor for full analysis.

In studies of uncultured HL, cytogenetic analyses have been complicated by the fact that so few cells are dividing. In LP-HL most mitoses have proved to be normal[78]. In Rowley's series of HL only 4/25 yielded clones[78] and these could not be completely karyotyped. Most recent series have yielded conflicting data. Schlegelberger et al[69] studied 21 patients using combined immunophenotyping and karyotyping and found aberrant clones in 12 - all in CD15/CD30 bearing cells. Most of these were complex abnormalities, 2 appeared to involve only the sex chromosomes. In contrast Ladanyi et al[79] in the largest series to date of 95 cases found that 81% had normal karyotypes and of the 19% which should any karyotypic abnormalities 3/13 were non clonal; thus results from cytogenetic investigations are variable.

Specific translocations have not been linked to HL and although *bcl-2* protein is produced by the RS-H cells attempts to link *bcl-2* production to the t(14;18) has been disappointing[80] and there is some doubt as to whether t(14;18) bearing cells when found are actually malignant[81].

The Ig heavy chain rearrangements were found in most cases studied. Although confined to the CD30+ve population (L25, L17) they were seen in both in one case (L20), the sensitivity of the test and the known failure rate of the sorting procedure would be able to account for this. The presence of these and the ability to grow

the lymphoma in a B cell culture system support a B cell origin for this disease.

Molecular techniques using Southern blotting to analyse the configuration of Ig and T cell receptor (TCR) rearrangements have produced heterogeneous results ranging from absence in the majority of studies to clonal rearrangements in some[23,82-87]. This may, however, represent the paucity of tumour cells. In addition, the distinction between a DJ and VDJ rearrangement can not be made because of the use of J_H probes. Another disadvantage of using Southern blots is that relatively large amounts of undegraded DNA are required. A PCR based technique overcame this problem and was able to detect only a VDJ rearrangement that was lineage specific and enabled the clonality rearranged V_H genes to be sequenced allowing detection of somatic mutations in the V regions, thus permitting the distinction between pregerminal (naive) or post germinal (memory and effector B cells).

Recently, microdissection of sections of HL and the taking of individual RS-H cells characterised by their expression of CD15 and CD30 with the use of a PCR based technique to amplify the V_H region has revealed that cells in a particular patient come from a single clone (3 patients studied)[88]. This may be because of a selective growth advantage of the malignant cells. However, in all cases studied a band was present prior to culture suggesting the method to be sufficiently sensitive to pick low levels of clonal populations. The ability of the molecular technique used to

identify a band in most cases studied contrasts with the inability of others[89] to find such evidence when looking at uncultured samples.

In the absence, of microdissection, using a PCR based technique to study Ig rearrangements in paraffin embedded samples only 1/36 showed Ig rearrangement[89]. Confirmation of the clonal nature of RS cells has been carried out using chromosome specific and satellite DNA probes from different biopsies of the same patients. Interestingly, 20% in the RS cells showed diploidy whilst the rest were aneuploid or tetraploid[74].

The failure to find karyotypic evidence of a specific translocation associated with any subtype of HL has encouraged the use of a PCR based strategy to look for the t(2;5) - found in some cases of anaplastic large cell lymphomas[90]. In one series this was found in 11/13 NS cases studied[91], although it has not been confirmed by others[92].

Cytocentrifugation specimen analysis was crucial to the interpretation of the findings. It confirmed the success of the cell sorting using CD30. The presence of RS cells and mononuclear H cells and the ability of the LP subtypes to differ in culture from the others in terms of the relative frequency of these cells and the large numbers of blast-like cells confirmed the difference in culture between LP and non LP subtypes. The number of RS type cells in culture was similar to the proportion seen *in vivo* - in a lymph node with HL and much lower than that suggested by the

proportion of CD15 and CD30. L18 (MC) was unusual in that although binucleated cells were present in culture, the majority of cells were plasmacytoid as the culture progressed, a finding not seen in other cases. L20 represented an unusual case in that the patient relapsed with LP-HL 22 years ago and the relapse specimen at the time of biopsy showed sheets of L+H cells representing clonal progression to high grade B cell lymphoma. The presence of light chain restriction in culture with the persistence of the Ig heavy chain clone supports the fact that this is one disease.

The participation of T cells in the growth of HL as opposed to them being 'bystanders' or indeed fighting the disease is suggested by the fact that the CD30 ligand (CD30L) is present on T cells and the binding[24] to CD30 leads to differentiation and proliferation of CD30 bearing cells. This has been seen in HL and cell lines with T cell phenotype and may be a good argument not to T cell deplete prior to culture. Indeed, the one case where T cell depletion was not carried out because of the poor viability of the cells (L28) was able to proliferate very well without evidence of T cell overgrowth.

The number of tumours judged to be EBV positive by LMP status is in keeping with general findings - in particular both LP cases were LMP-ve. The suggestion that primary culture of Hodgkin's lymphoma of a variety of subtypes both EBV positive and negative is indeed possible using this system can be inferred from the total evidence presented. The strongest comes from FACS analysis

and cytocentrifugation specimens. Whilst FACS gives quantitative information on the percentage of CD15-ve and CD30+ve cells cytocentrifuged slides can confirm the presence of RS/L+H cells. It is of course not possible to say whether the bands obtained by PCR are from these cells but the fact that they were not present in benign tissue cultured, supports the fact that they came from the malignant clone(s).

In summary, the IL3/IL10 stromal cell system appears to support the growth of Hodgkin's lymphoma of both LP and non LP subtypes as suggested by morphology, flow cytometry and IgH heavy chain rearrangement.

CHAPTER 7

THE CD80 ANTIGEN AND ITS APPLICATION TO IMMUNOTHERAPY

7. THE CD80 ANTIGEN AND ITS APPLICATION TO IMMUNOTHERAPY

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7.1 Introduction

The CD80 antigen (B7-1) is one of a recently recognised group of accessory antigens which are considered to be very important in the eliciting of a T cell immune response. This chapter reviews the role of CD80 in immune regulation and describes the prevalence of CD80 expression in low grade B cell lymphoma as assessed by flow cytometry. Induction of CD80 expression in the stromal cell culture system is carried out using IL4 and comparisons with the results using IL3/IL10 are made. Assessment of these cells' immunogenicity by a primary mixed lymphocyte reaction is described and the results of autologous and allogeneic T cell responses are presented.

7.2 CD80 and Immune Function

The CD80 antigen[93], previously called B7, was first described in 1987 by Freedman et al[94]. Then it was considered to be B cell restricted and initial work suggested that it appeared in B cells within 24hrs of *in vitro* activation. It was found in a subset of B lymphocytes but not in T cells or monocytes - in addition it was not detected in leukaemia or lymphomas of T cell origin. It was found in some B-CLL, and prolymphocytic leukaemia as well as diffuse histiocytic lymphomas, but not in myeloma. Several cell lines expressed it - including Raji, Daudi and Ramos.

The antigen is a member of the immunoglobulin (Ig) superfamily[95] - a type 1 protein - 262 amino acids long with extracellular transmembrane and intracellular regions. The extracellular region contains two Ig domains - the first resembling the variable region and the second the constant domain. The gene encoding CD80 has been mapped to chromosome 3q13-q23[96]. The original ligand described for CD80 was CD28[97,98] and this is present on T cells and mediates adhesion with B cells by interacting with CD80[98]. The binding leads to IL2 synthesis by the T cells[94]. It was subsequently found by Linsley et al in 1991 that another receptor for CD80 existed, this was called CTLA4 and was identified by screening a murine cytolytic T cell cDNA library[100]. The gene for the human counterpart was cloned to the same region as CD28 (2q33-34) and the proteins showed some homology. Studies were undertaken using a fusion of the CTLA4 extracellular domain with an Ig Cγ1 chain to make CTLA4 Ig. CTLA4 Ig bound CD80 more avidly than CD28[100]. Studies

looking at the inhibitory role of antibodies against CD80 and the CTLA4 Ig found a difference in blocking ability - CTLA4 Ig - completely blocked T cell proliferation mediated by CD80 whilst a specific anti-CD80 antibody (B7-24)[101] was unable to do this. This suggested that more than one ligand for CTLA4 existed.

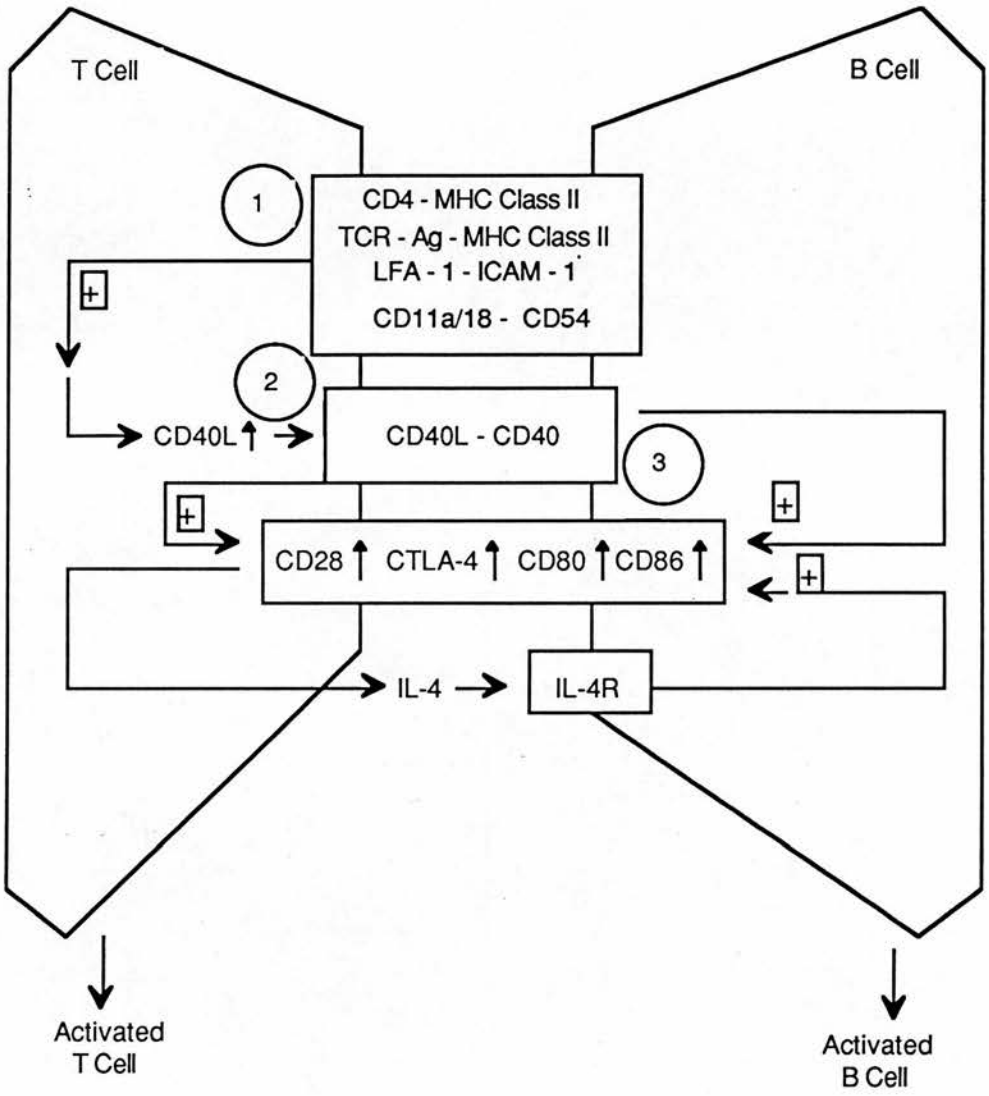
A second ligand was subsequently found termed B7-2[102] by one group and B-70 by another[103] - it was found to be the same as CD86[104]. This is a 70kd protein and there was evidence that it was also a member of the immunoglobulin superfamily which was found on resting monocytes, dendritic cells and on activated T, NK and B lymphocytes[103]. The gene mapped to the same region as CD80[96]. CTLA4 bound to CD86 as well. Upregulation occurred within 24hrs[105], compared to CD80 which took 48-72hrs. A 3rd molecule B7-3 has more recently been identified[106]. This upregulated over the 48-72hr period and was recognised by BB-1 - the original antibody[106] which recognised CD80. This may have lead to differences in staining patterns when this antibody was used[95], other antibodies producing different results. One, MAb 104 recognised CD80 on B cells and also on transformed T cell lymphomas as well as $^{3}/_{5}$ CLL, $^{5}/_{5}$ follicle centre lymphomas (FCL)[107]. Another, B7-24[101] which recognised CD80 has been shown to block mixed lymphocytes reactions with T cells. Although not initially found on resting monocytes - activated ones have been found to express CD80[54] and suppression of macrophage activation by IL10 appears to act by downregulation of CD80[54]. It has also been found in Reed Sternberg cells[108,109] where it is thought to be important in T cell stimulation and

induction of cytokine synthesis and suggest that T cell may be actively involved in Hodgkin's lymphoma rather than bystanders.

The most potent suppressor of T/B cell responses affecting the B7 system is CTLA4 Ig as the affinity of CTLA4 is for all members of the B7 family.

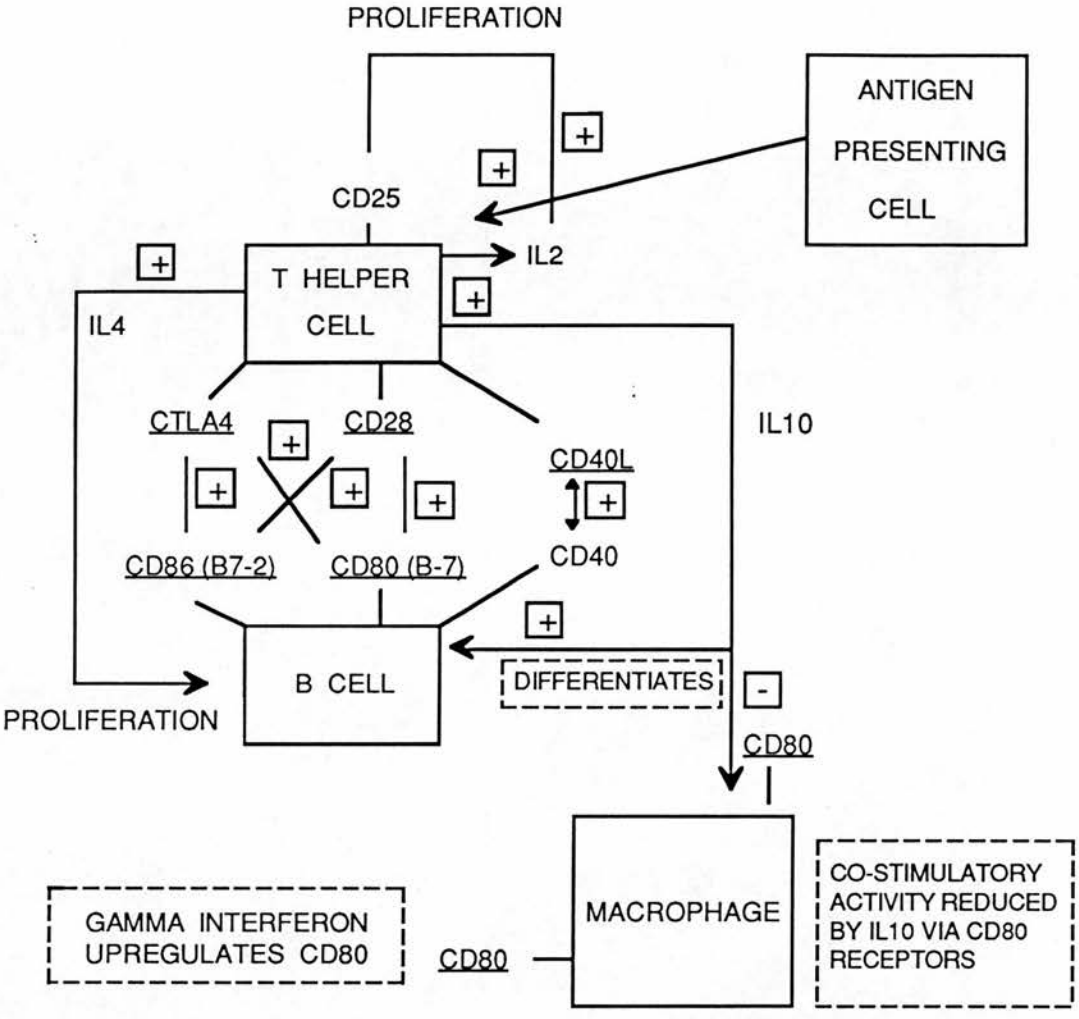
The interaction between CD40, CD80/CD86 and CTLA4/CD28 is complex. CD40 on B cells, binding to the CD40L on T cells stimulates CTLA4 production as well as CD28 - and this induces CD80/CD86 on the B cells. This leads to IL4 production by the B cells which upregulates CD80 expression[28]. In addition, ligation of CD80 with CD28/CD86 leads to CD40L expression and the synthesis of IL4[110]. IL2 also has been shown to upregulate CD80[111] (see Figures 1 and 2).

Figure 1: Interaction between T and B cells showing how CD40 and IL4 mediate a positive feedback loop to induce CD80/CD86 expression



TCR - T cell receptor
MHC - Major histocompatibility complex
CD40L - CD40 ligand
IL4R - IL4 receptor

Figure 2: The T/B/Macrophage Interactions and their regulation by cytokines and CD80 System



This system of positive feedback in B lymphocytes was a rationale for investigating CD80 expression in low grade B cell lymphomas cultured on anti-CD40 stromal cell system.

Binding of CTLA4 and CD28 to CD80 is via the complementary determinatory region (CDR1) and CDR3 analogous regions which are conserved in both molecules[104]. Residues in the V domain of CD80 have been implicated in the functional interactions with CD28 and CTLA4. One such residue is conserved in CD86.

Mutations in this domain lead to failure of upregulation of IL2 production by phytohaemagglutinin (PHA) stimulated Jurkat cells[112].

7.3 The role of CD80 and other adhesion molecules in B/T cell interactions

For antigen to be presented successfully to T cells 3 signals are required:- recognition, adhesion and costimulation (see Figure 3).

1) The antigen must be presented with Class I (for intracellular protein) or Class II (Major Histocompatibility antigens) to the T cell receptor/CD3 complex - coupled to Class I - a CD8 molecule is needed whilst coupled to Class II a CD4 molecule is required.

2) Adhesion: Several adhesion molecules are required:- the antigen presenting cell possession LFA-1 (CD11a/18), CD54 (ICAM-1) and CD58 (LFA3) - the T cell has corresponding counter ligands LFA-1 and CD54 make a pair as do CD58 and CD2 and CD106 (VCAM) and CD49 d/29 (VLA-4).

3) Costimulation: In addition an accessory signal is required (costimulatory signal). Costimulating signals: CD58, CD54, CD40, CD80, CD86 and their counter ligands[113].

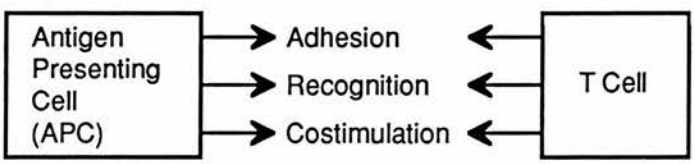


Figure 3

HLA-ABC HLA - DR/DP/DQ	Recognition	CD3/TCR/CD8 CD3/TCR/CD4
APC CD54 CD11a/18 CD106 CD58	Adhesion	T Cell CD11a/18 CD54 CD49d/29 CD2
CD58 CD54 CD40 CD80 CD86	Costimulation	CD2 CD11a/18 CD40L CD28 or CTLA4 CD28 or CTLA4

Failure to deliver a costimulating signal leads to the T cell failing to produce IL2 which can act in an auto/paracrine fashion to induce T cell proliferation.

Some of the adhesion molecules listed can act as costimulating ones - whilst CD54 can induce an allogeneic response, it can be inhibited by cyclosporin and it fails to lead to IL2 synthesis - unlike CD80[113].

In addition - a rechallenge of T cells - leads to a secondary response only with CD80. With CD54, anergy results which can be overcome by the addition of exogenous IL2 suggesting that T cells respond normally when correctly stimulated.

7.4 The role of CD80 in tumour vaccines

The most exciting evidence for the crucial role of CD80 in the production of tumour vaccines came from work by Townsend and Allison[114]. In a murine model they grew a murine melanoma cell line in athymic mice, this was then taken and transfected with CD80. The cells were equally tumourigenic in athymic mice, but in intact mice only the CD80 naive tumour grew aggressively with most CD80 expressing tumours disappearing or becoming static. Vaccination with fragments of CD80 expressing cells protected most mice against challenge with CD80 naive tumour cells. T cell depletion allowed CD80 expressing tumour to grow rapidly when CD8 cells were depleted but not when CD4 cells were depleted. Thus, providing costimulation by expression of CD80 protects against subsequent challenge by CD80 naive cells and this is mediated by CD8 T cells.

Cotransfection with CD80 and IL7 is able to induce CD28 and CD25 (IL2 receptor) in T lymphocytes. In CD80 transfected tumours only, the T cell response was mostly CD28+ve; CD25-ve; whilst in IL7 transfectants it was the other way around suggesting the two to be acting via different pathways[112]. In similar experiments immunising mice with transfected cells and then challenging with CD80/IL7 immune cells suggested that the CD80/IL7 cells provided better protection than either on its own. Abrogation by irradiation of the cells suggested that live tumour cells would be required for such an approach[112].

7.5 Evidence for the role of CD80 in induction of immune tolerance

Perhaps the most immediate use for manipulation of CD80 comes from studies in immunosuppression. Blocking with CTLA4 Ig especially, is able to depress mixed lymphocyte reactions (MLR)[105] and anti-CD80 (B7-24) has been able to depress a primary MLR to a greater degree than cyclosporin, the combination showing almost complete inhibition[115].

Thus the first role of clinical importance may be to modify acute graft versus host disease or perhaps to prevent it altogether[113].

7.6 CD80 in low grade B cell Lymphoma

In order to assess the role of CD80 augmentation in the immune response to low grade lymphoma, it was first necessary to look at expression in some samples since up till then the data were somewhat sparse.

7.6.1 *Anti-CD80 Ig studies*

2 anti-CD80 Igs were used in the studies described:

a) BB1 - commercially from Beckton Dickinson. This was described by Linsely et al[98] and bound to Raji cells. It is an $IgG_1\kappa$ Ig.

b) B7-24 (provided by Innogenetics) described by de Boer et al[101]. It was obtained by fusion with splenocytes from a mouse immunised with SF9 insect cells expressing CD80. It is an $IgG_{2a}\kappa$ Ig.

Titration and Comparisons of Igs

Both antibodies were titrated against a Raji cell line. The optimal dilution for BB-1 was found to be $^1/_{160}$ - $^1/_{320}$ by FACS analysis - starting concentration of 100µg/100µl.

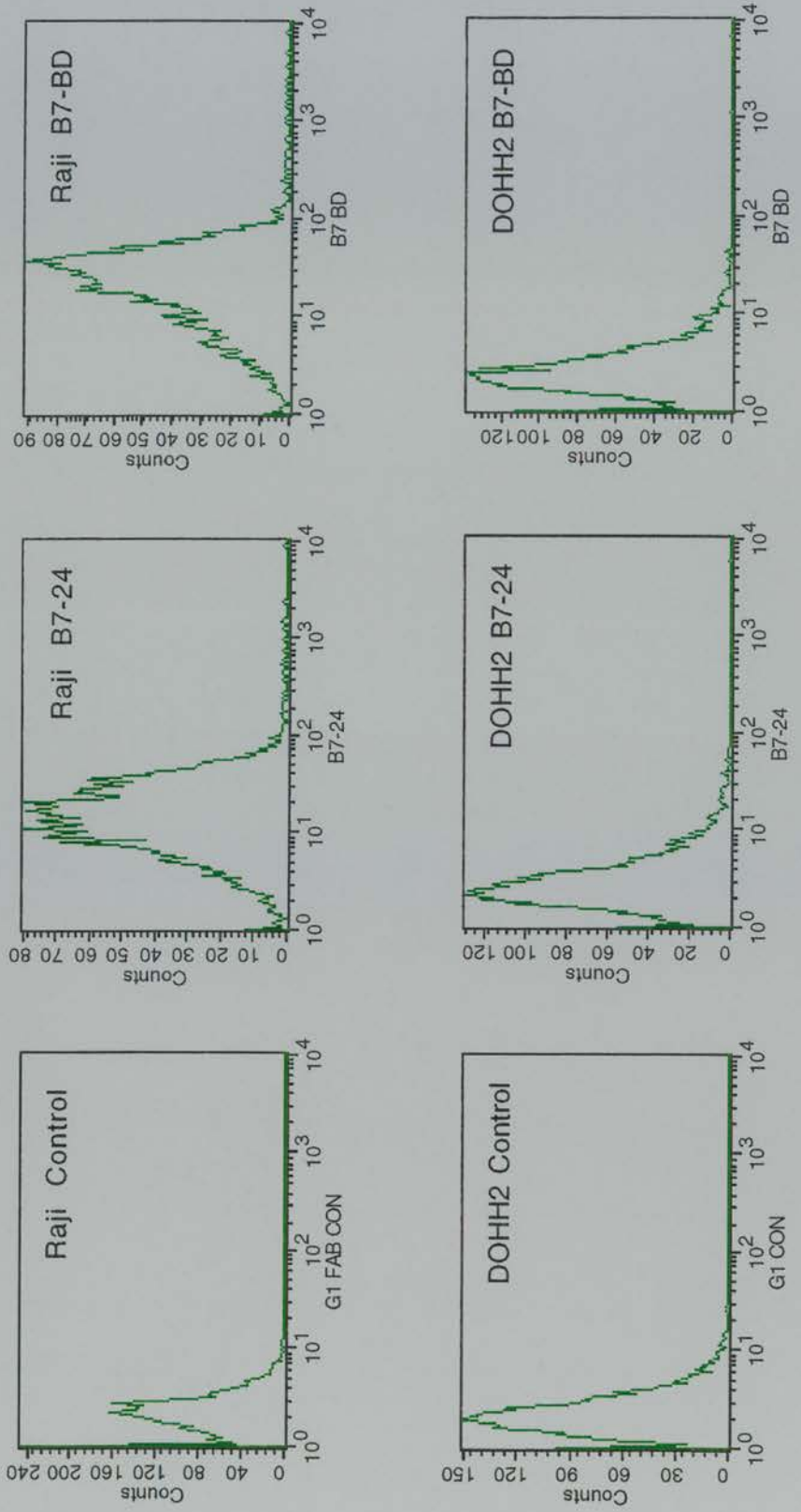
The optimal dilution of B7-24 was also found to be $^1/_{160}$ - $^1/_{320}$ - starting concentration 200µg/100µl.

The antibodies required a 2nd layer of FITC rabbit $F(ab)_2$ anti-mouse Ig (Dako) at a dilution of $^1/_{40}$.

Comparison of the 2 antibodies in the Raji cell line

Both antibodies produced positive results, however the profiles were not identical suggesting binding to different epitopes. The $DOHH_2$ cell line did not stain for either.

Figure 4: CD80 expression in Raji and $DOHH_2$ cell lines as measured by two antibodies B7-24 and BB-1 (B7-BD)



Cell Cycle Analysis

As activation of B cells is required to upregulate CD80. Raji cells were labelled with Hoechst 33242 and cells were analysed using a FACStar and separated by DNA content and CD80 expression. No variation in CD80 expression with cell cycle was found.

In view of the small numbers of low grade lymphomas studied for CD80 expression a larger series was studied by flow cytometry. 12 low grade lymphomas (9 follicle centre cell, 2 B-small lymphocytic, 1 splenic marginal zone B cell lymphoma). The lymphoma suspensions were prepared as described before and the immunophenotyping was carried out using fresh tissue and then following thawing to make sure that the freezing and thawing procedure had not affected expression. Phenotyping used BB-1 (Beckton Dickinson).

7.6.2 *Expression of CD80 in cultured cells*

B7-24 was used to assess CD80 expression in these cells as the presence of anti-CD40 (IgG_1) meant that when the 2nd layer of rabbit anti-mouse was applied it would bind to the anti-CD40 leading to a false positive result. Using B7-24 (IgG_{2a}) allowed a specific FITC-anti-mouse IgG_{2a} to be used which did not react with IgG_1 immunoglobulins allowing measurement of CD80.

7.6.3 *Expression of Adhesion Molecules*

As various adhesion molecules are important in costimulation it was decided to measure expression of CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA3) (all Serotec) on lymphoma cells prior

to and post culture. In addition, HLA-DR (Beckton Dickinson) was measured to ensure that all the necessary molecules to all these measurements were carried out following T cell and IgD cell depletion (see 3.6).

7.6.4 *Lymphoma Cells*

Lymphoma cells were cultured using IL4 (46μg/L) and anti-CD40 in the stromal cell system previously described with the specimens having first undergone T and IgD bearing cell depletion using Dynabeads.

7.7 Results

7.7.1 *Initial phenotyping of samples*

(a) Fresh

Immunophenotyping of fresh samples revealed poor expression of CD80. The median proportion of cells expressing CD80 compared to control was 9% (standard deviation (SD) 24, range 2-89%). There was no evidence of a discrete CD80 positive population of the cells.

(b) Frozen

Phenotyping of the same lymphoma cells following thawing using the antibody B7-24 revealed a median expression of 8% (SD16) range 1-59%.

7.7.2 *Cell Viability*

Viability of resuspended cells was assessed using 0.2% trypan blue exclusion, a median viability of 95% (SD3) was seen in the 12 samples studied.

7.7.3 *Confirmation of T and IgD bearing cell depletion and clonality*

The use of paramagnetic beads reduced the proportion of T cells from a mean of 34% (SD20) to 8% (SD7) and IgD bearing cells from 22% (SD17) to 6% (SD7). The cells showed light chain restriction using 2-colour staining (details of individual results are shown in Tables 1 and 2).

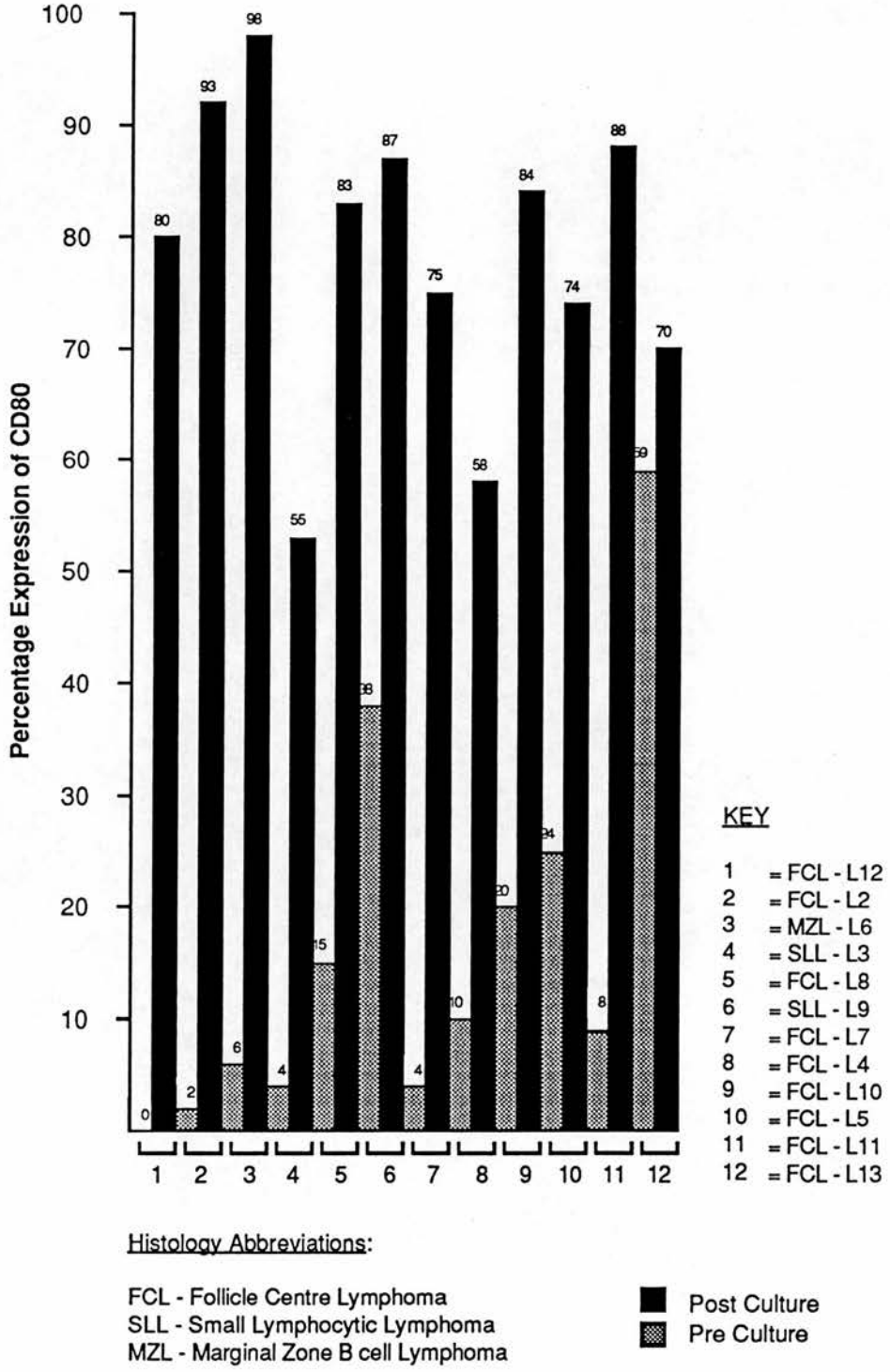
7.7.4 *Bcl-2 and adhesion molecule expression*

The cells showed low but significant expression of *bcl-2*. Median expression of CD11a, CD54 and CD58 was 34% (SD19), 50% (SD17) and 84% (SD17) respectively.

7.7.5 *Analysis of cells harvested after 10-13 days - CD80 and adhesion molecule expression*

Increased expression of CD80 was shown by a shift in median channel fluorescence. Overall expression increased 16.1 (SD22) to 62.7 (SD17) per culture (see Figure 5).

Figure 5: Expression of CD80 (B7-24) compared to IgG2a control isolated low grade lymphoma B cells before and 10-13 days after growth in the IL4/anti-CD40 stromal cell culture system



Details of individual lymphomas are shown in Table 2. During culture a B cell proliferation occurred without evidence of T cell overgrowth. The cells continued to show light chain restriction on FACS analysis but there was a shift in phenotype with an increase in CD19+ve cells and an upregulation in the adhesion molecules studied. Expression of CD11a, CD54 and CD58 increased to 58% (SD22), 92% (SD14) and 99% (SD2) respectively.

In 4 lymphomas studied for HLA-DR expression this was high at the end of the culture period.

Table 1

CHARACTERISTICS OF CULTURED CELLS AT DAY 10-13									
Sample	Histology	CD3	IgD	CD19	Light Chain	CD80	CD11A	CD54	CD58
1 - L12	FCL	3	40	53	61κ	80	81	N/A	99
2 - L2	FCL	9	2	68	95κ	93	87	96	99
3 - L6	MZL	12	49	82	63κ	98	53	98	100
4 - L3	SLL	4	3	51	99κ	55	30	96	97
5 - L8	FCL	6	33	58	69κ	83	64	90	98
6 - L9	SLL	14	2	47	98κ	87	49	66	95
7 - L7	FCL	2	41	57	95κ	75	31	93	97
8 - L4	FCL	17	10	25	76κ	58	39	68	94
9 - L10	FCL	7	18	50	78κ	84	35	57	100
10 - L5	FCL	19	65	59	61κ	74	89	98	99
11 - L11	FCL	5	18	71	75κ	88	61	91	99
12 - L13	FCL	9	50	65	N/A	70	89	N/A	99

Table 2

PRE-CULTURE B CELL CHARACTERISTICS FOLLOWING IGD AND CD3 BEARING CELL DEPLETION									
Sample	Histology	CD3	IgD	CD19	LIght Chain	CD80	CD11A	CD54	CD58
1 - L12	FCL	13	13	62	90κ	0	36	68	87
2 - L2	FCL	7	0	27	97κ	2	33	64	62
3 - L6	MZL	2	2	38	65κ	6	53	37	42
4 - L3	SLL	7	1	84	99κ	4	32	20	86
5 - L8	FCL	5	2	35	92κ	15	61	49	72
6 - L9	SLL	14	1	71	99κ	38	26	49	72
7 - L7	FCL	4	6	45	96κ	4	22	54	96
8 - L4	FCL	6	1	13	95κ	10	12	44	43
9 - L10	FCL	3	8	70	97κ	20	45	51	84
10 - L5	FCL	20	3	12	83κ	24	23	23	65
11 - L11	FCL	2	16	70	97κ	8	79	70	77
12 - L13	FCL	16	24	30	74κ	59	64	75	N/A

Histology Abbreviations:
FCL - Follicle Centre Lymphoma
SLL - Small Lymphocytic Lymphoma
MZL - Marginal Zone B Cell Lymphoma

7.7.6 *Discussion*

The stromal cell culture system offers the potential to enhance expression of certain antigens which may be important to the costimulation of T lymphocytes.

In the past, a drawback has been the apparent change in phenotype with a loss of mature cell antigens and the development of blast like morphology[34], this has been seen in all lymphomas studied here. However, the alteration and inter lymphoma variation of CD19 and IgD bearing cells does not

appear to affect CD80 expression or the expression of the adhesion molecules studied. Thus, IL4 can induce CD80 expression in benign[111] and malignant B cells; cross linking of CD40 alone can also induce expression of it in benign cells.

Having shown that the IL4/anti-CD40 system can induce CD80 expression in cultured low grade lymphoma it was necessary to show that these cells were: (1) more immunogenic than the uncultured lymphoma cells and (2) that the patient's own T cells would respond to them.

The IL3/IL10 system whilst producing different phenotypes from those found with IL4 had not been shown to induce CD80 expression and cells grown on this system were tested for immunogenicity. It was decided in the first instance to use a primary mixed lymphocyte reaction to assess immunogenicity of cultured lymphoma cells.

7.8 <u>The use of a primary mixed lymphocyte reaction to evaluate the immunogenicity of cultured low grade B cell lymphoma</u>

The combination of antigen presenting cells with T cells should lead to T cell proliferation if the right signals - HLA Class I and II, adhesion and accessory costimuli are present. To assess whether a T cell response would be mounted to cultured low grade lymphoma - lymphoma cell suspensions were taken and incubated with T cells (allogeneic and where available autologous) and the results compared to the same lymphomas cultured using

IL3/IL10 or IL4 in the stromal cell system using anti-CD40. On this occasion no T cell depletion was attempted.

T cell proliferation was assessed by the uptake of ^{3}H-thymidine with radionuclide decay being proportional to T cell proliferation. Sampling was carried out at several intervals to see if the response increased with time. Various controls were introduced and all experiments were carried out in triplicate.

Materials and Methods - the mixed lymphocyte reaction:-

Preparation of presenting cells (stimulators):- 5 lymphomas (L2, L4, L11 and L13 - all FCL) were taken. Uncultured lymphoma cells, lymphoma cells cultured in the IL3/IL10 anti-CD40 stromal cell system and in the IL4 anti-CD40 stromal cell system and adherent mononuclear cells prepared from peripheral blood acted as stimulators for the MLR. To culture the lymphomas a 24 well culture plate was used and 10^{6} viable cells were plated out per well, 8 wells were used for each culture system. The cells were harvested at day 10 and immunophenotyped for CD80 expression (using B7-24) and for CD11a, CD54 and CD58 and HLA-ABC and HLA-DR. Cells were taken from uncultured lymphomas (same) and similarly immunophenotyped. To ensure they did not proliferate they were treated with mitomycin C (50μg/10^{7} cells/ml) for 30 minutes at room temperature followed by centrifugation (5 minutes, 600g, 25^{o}C) and washing five times in minimal essential medium.

Preparation of responding cells

50ml of whole blood was taken, and diluted to 200ml in PBSA. The diluted blood was centrifuged on a Ficoll Hypaque gradient (1,000g, 25 minutes, 25°C) the mononuclear layer was removed and washed twice in PBSA. The monocytes were then removed by resuspending the mononuclear cells in RPMI supplemented with 5% FCS and placed in a 250ml Falcon flask (B.D.) rested horizontally at 37°C for $1^1/_2$ hours. The flask was then stood upright for 5 minutes leaving behind the adherent monocytes stuck to the flask.

Setting up the MLR

The stimulators were placed in 96 well round bottomed plates (Falcon 8077) 3 X 10^4 cells were placed in each well in 100µl of RPMI with 10% FCS. To them were added 10^5 responding cells (blood lymphocytes) in 100µl of RPMI with 10% FCS. If no responding or simulators were required then 100µl of RPMI and 10% FCS without cells was added. The arrangement is shown in Figure 6.

Figure 6: Set up of 96 well plates

	1-3	4-6	7-9
A	IL3/IL10 Cultured Cells + Allogeneic + Allogeneic Responders	IL3/IL10 Cultured Cells + Autologous Responders	IL3/IL10 Cultured Cells Alone
B	IL4 Cultured Cells + Allogeneic Responders Responders	IL4 Cultured Cells + Autologous Responders	IL4 Cultured Cells Alone
C	Uncultured Lymphoma Cells + Allogeneic Responders	Uncultured Lymphoma Cells + Autologous Responders	Uncultured Cells Alone
D	Allogeneic Responders + Special Positive Control	Autologous Responders + Special Positive Control	Special Positive Control Alone
E	Allogeneic Responders Alone	Autologous Responders Alone	Nil

The cells were pulsed with ^{3}H-thymidine for the last 6 hours of culture at 3, 5 and 8 days post incubation. The cells were then frozen and counted. A different plate was used for each period of incubation.

(1) *Immunophenotyping of lymphoma cells pre and post culture*:- Expression of the adhesion molecules CD11a, CD54, CD58 and the costimulatory molecule CD80 pre and post culture is shown in Table 3. HLA-ABC was found in all cells where measured pre and post culture. HLA-DR was present in a variable number of pre and post culture. Overall, CD11a, CD54 and CD58 altered in culture although no pattern was seen in the different culture groups. There was a tendency for CD11a expression to diminish in culture whilst CD58 tended to increase. CD80 expression increased in cultured

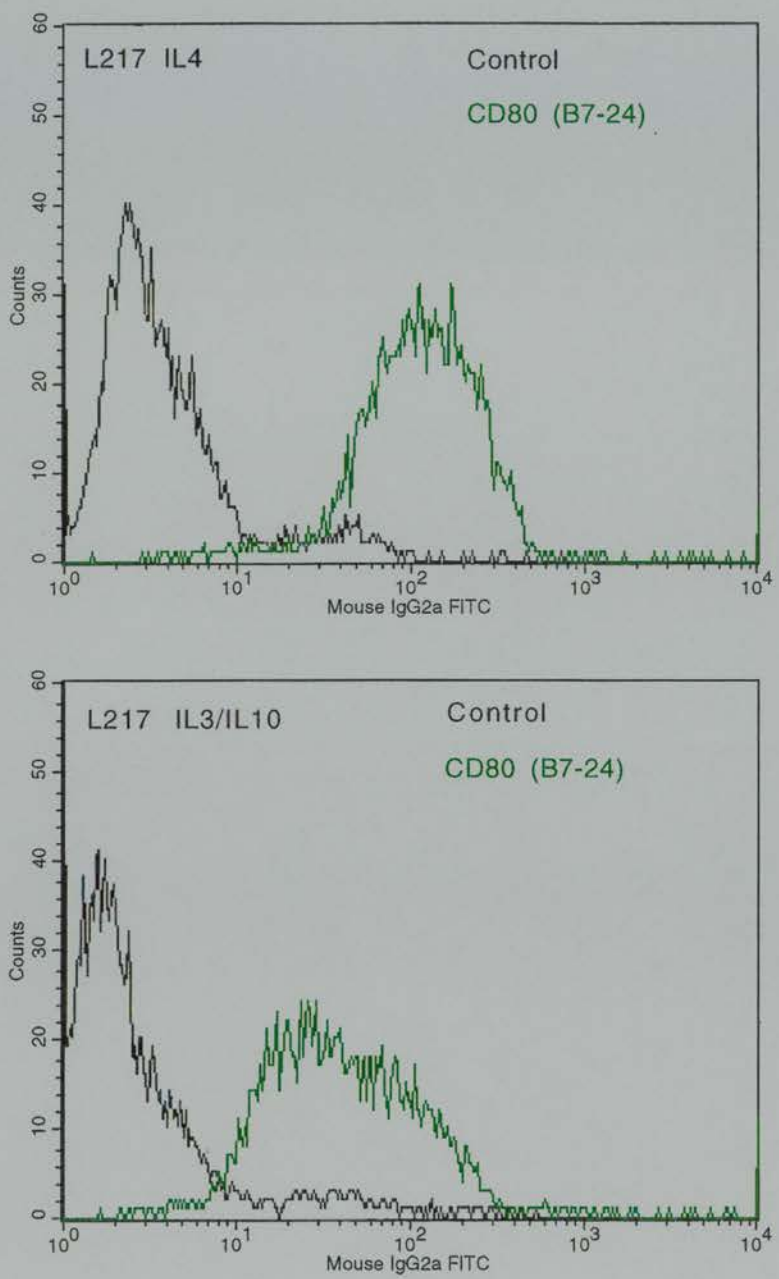
cells and this was more marked with IL4. An example of CD80 expression in L2 in the two cytokine systems is shown in Figure 7.

Table 3: Results

Lymphoma		CD11a	CD54	CD58	HLA-ABC	HLA-DR	CD80
L2	Uncultured	65	41	88	100	83	2
L2	IL3/IL10	38	44	67	99	63	37
L2	IL4	32	31	99	99	78	92
L4	Uncultured	12	44	43	100	32	2
L4	IL3/IL10	N/A	N/A	N/A	100	74	72
L4	IL4	39	68	94	100	83	92
L11	Uncultured	53	11	26	100	63	4
L11	IL3/IL10	89	67	80	100	81	66
L11	IL4	31	36	61	100	82	71
L12	Uncultured	73	40	87	100	76	1
L12	IL3/IL10	46	50	66	98	57	18
L12	IL4	41	61	75	99	67	24
L13	Uncultured	73	14	26	N/A	N/A	12
L13	IL3/IL10	28	8	53	N/A	N/A	21
L13	IL4	39	6	71	N/A	70	41

N/A = Not available

Figure 7: **CD80 expression in cultured lymphoma cells grown in IL3/IL10 or IL4**



(2) *Mixed lymphocyte reactions*:-

In all cases the responding lymphocytes were reactive, judged by the response to the adherent cells (positve control), however the background counts varied somewhat from one experiment to another.

In all cases the allogenic and autologous responses were greater when cells were cultured in IL4 than in IL3/IL10, with culture in IL4 these results were substantially higher than the response to the uncultured cells. In one case, using IL3/IL10 there was little difference between the cultured group and the uncultured group as far as allogeneic responses were concerned (L12).

Table 4: Results of L1108

L1108	Day 3	Day 5	Day 8
IL3/IL10 + Allo	12,350 ± 3,100	40,100 ± 4,300	177,500 ± 45,700
IL4 + Allo	25,900 ± 3,500	69,500 ± 4,100	380,500 ± 46,700
IL3/IL10 + Auto	1,300 ± 340	2,900 ± 700	1,600 ± 340
IL4 + Auto	4,700 ± 130	2,900 ± 200	3,200 ± 550
IL3/IL10 Alone	1,500 ± 500	2,700 ± 1,300	1,300 ± 320
IL4 Alone	3,600 ± 400	2,200 ± 200	1,300 ± 100
Uncultured + Allo	5,400 ± 100	15,700 ± 2,300	54,800 ± 23,600
Allo Alone	1,700 ± 200	2,100 ± 1,000	9,200 ± 5,000
Uncultured + Auto	1,200 ± 100	1,200 ± 200	1,000 ± 100
Auto Alone	900 ± 200	1,100 ± 100	600 ± 100
Uncultured Alone	1,000 ± 100	1,000 ± 50	1,350 ± 100
Special +ve Only	1,700 ± 100	2,100 ± 300	2,600 ± 400
Allo Responders + Special +ve Control	13,600 ± 1,000	27,000 ± 4,000	107,800 ± 12,000
Auto Responders + Special +ve Control	1,500 ± 100	2,100 ± 200	10,700 ± 2,600

Conclusion: MLR worked - special +ve. controls and allogeneic or autologous lymphocytes responded. Allogeneic responses - superior to autologous. IL4 >> IL3/IL10. Autologous with IL3/IL10 - no greater than when cultured cells. Autologous with IL4 - weak. Although greater than IL3/IL10.

There was evidence that cultured cells much more immunogenic than uncultured

Table 5: Results of L13

L1303	Day 3	Day 5	Day 8
IL3/IL10 + Allo	5,800 ± 700	8,900 ± 400	10,900 ± 2,200
IL4 + Allo	4,200 ± 600	10,900 ± 2,800	10,700 ± 2,200
IL3/IL10 + Auto	1,800 ± 200	9,800 ± 1,700	
IL4 + Auto	5,000 ± 700	5,200 ± 1,000	
IL3/IL10 Alone	1,800 ± 200	2,400 ± 700	3,100 ± 800
IL4 Alone	1,800 ± 200	2,000 ± 200	2,300 ± 300
Uncultured + Allo	2,600 ± 200	3,600 ± 300	3,800 ± 800
Uncultured + Auto	1,000 ± 100	5,300 ± 1,000	
Uncultured Alone	1,500 ± 600	3,100 ± 400	1,700 ± 200
Special +ve Alone	2,300 ± 1,000	1,900 ± 200	1,400 ± 100
Allo + Special +ve	4,200 ± 100	6,100 ± 300	7,600 ± 300
Auto + Special +ve	550 ± 100	4,700 ± 800	
Auto Alone	4,070 ± 100	2,300 ± 400	
Allo Alone	3,000 ± 200	2,300 ± 100	2,700 ± 200

Conclusion:- MLR worked, the allogeneic responses were superior to the autologous ones. In this case although responses occurred earlier in the cultured cells they were eventually matched by those in the uncultured group. IL4 cultured cells elicited stronger responses than IL3/IL10.

Table 6: Results of L2

L218	Day 3	Day 5	Day 8
IL3/IL10 + Allo	6,000 ± 1,600	8,200 ± 1,600	8,000 ± 1,400
IL4 + Allo	8,000 ± 900	15,200 ± 2,300	14,500 ± 2,800
IL3/IL10 + Auto	7,000 ± 800	4,800 ± 1,000	6,800 ± 1,700
IL4 + Auto	9,200 ± 500	13,100 ± 1,900	14,700 ± 2,800
IL3/IL10 Alone	2,500 ± 200	2,100 ± 700	4,800 ± 900
IL4 Alone	2,500 ± 300	1,900 ± 700	4,200 ± 300
Uncultured + Allo	3,200 ± 300	6,300 ± 700	6,800 ± 1,100
Uncultured + Auto	3,600 ± 100	4,100 ± 300	5,200 ± 100
Uncultured Alone	1,400 ± 200	1,400 ± 300	1,700 ± 300
Special +ve Only	1,400 ± 200	1,400 ± 200	1,900 ± 200
Allo + Special +ve Control	2,900 ± 300	5,000 ± 1,100	8,700 ± 800
Auto + Special +ve Control	3,500 ± 100	6,700 ± 300	11,400 ± 1,000
Auto Alone	2,800 ± 20	2,800 ± 50	3,300 ± 100
Allo Alone	2,200 ± 500	2,100 ± 100	3,100 ± 10

Conclusion:- MLR worked. Cultured cells lead to stronger respones than uncultured. Allogeneic and autologous responses were equivalent. IL4 cultured cells lead to stronger responses than IL3/IL10.

Table 7: Results of L4

L404	Day 3	Day 5	Day 8
IL3/IL10 Allo	15,600 ± 1,300	32,700 ± 1,400	129,900 ± 17,600
IL4 Allo	16,300 ± 5,800	58,500 ± 1,500	243,100 ± 19,800
IL3/IL10 Alone	2,300 ± 500	1,200 ± 100	1,600 ± 100
IL4 Alone	1,500 ± 100	1,400 ± 200	1,200 ± 200
Uncultured + Allo	5,200 ± 900	13,100 ± 1,700	40,500 ± 8,000
Uncultured Alone	1,000 ± 100	1,000 ± 50	900 ± 100
Allo + Special +ve Control	11,100 ± 1,100	22,500 ± 3,400	90,200 ± 8,200
Allo Alone	2,600 ± 500	5,800 ± 1,400	14,400 ± 8,000
Special +ve Control Alone	1,700 ± 100	2,100 ± 300	2,600 ± 400

Conclusion:- MLR worked. Cultured cells lead to stronger responses than uncultured cells. No autologous lymphocytes were available.

Table 8: Results of L12

L1205	Day 3	Day 5	Day 8
IL3/IL10 + Allo	300 ± 50	150 ± 50	50 ± 20
IL4 + Allo	650 ± 50	250 ± 100	450 ± 200
IL3/IL10 + Auto	650 ± 100	500 ± 300	950 ± 400
IL4 + Auto	1,700 ± 100	1,100 ± 400	4,000 ± 1,500
IL3/IL10 Alone	60 ± 10	10 ± 0	50 ± 30
IL4 Alone	150 ± 50	50 ± 10	20 ± 0
Uncultured + Allo	150 ± 50	40 ± 30	100 ± 50
Uncultured + Auto	450 ± 100	550 ± 150	5,500 ± 1,700
Uncultured Alone	30 ± 0	10 ± 0	20 ± 0
Special +ve Only	150 ± 0	100 ± 30	100 ± 50
Allo + Special +ve Control	600 ± 100	1,300 ± 200	13,900 ± 1,900
Auto + Special +ve Control	1,400 ± 100	4,800 ± 500	17,600 ± 2,900
Auto Alone	150 ± 100	350 ± 200	550 ± 500
Allo Alone	50 ± 0	20 ± 0	20 ± 0

Conclusion:- Counts generally low, but special positive controls confirmed that both allogeneic and autologous cells were capable of responses. Autologous responses were greater than allogeneic ones, although some responses with uncultured cells were equivalent to those using IL3/IL10 cultured cells.

7.9 Discussion

The experiments described suggested that there had been a change in the immunogenicity of the cultured cells as judged by a primary mixed lymphocyte reaction. The 5 follicle centre lymphomas all of which had low expression of CD80 prior to culture appeared unable to elicit a significant allogeneic mixed lymphocyte reaction particularly early on (day 3) although there had been a rise by day 8. The autologous responses in the 4 uncultured lymphomas studied were weaker than their allogeneic counterparts in 3 cases (L218, L1303 and L1108) but stronger in L1205 - a case where the allogeneic response was particularly weak despite the fact the allogeneic response in this case to the special positive control was strong - suggesting that no impairment of the response existed with the allogeneic lymphocytes. As these lymphoma cells possessed MHC Classes I and II and CD54; CD58 and CD11a and these can induce an allogeneic response[113] perhaps this is not surprising.

In culture, where these lymphoma cells had been induced to express CD80 in both the IL4/anti-CD40 system and the IL3/IL10 anti-CD40 system with continuing although varying expression of the other adhesion molecules the MLR would be expected to be stronger. In all cases CD80 expression was stronger in the IL4 group than the IL3/IL10 group, although CD54 appeared to be more expressed in the latter. The allogeneic responses to cultured cells in both groups were much stronger than in the uncultured, with the result in IL4 generally being stronger. As far as the autologous response was concerned a stronger response

was seen for the IL4 group in cases L1108, L218, L1205. In L1303 the responses were very similar.

In two cases the autologous responses were weaker than the corresponding allogeneic ones (L1108, L1303), equivalent in one (L218) and stronger in one (L1205). In one case (L218) the IL3/IL10 cultured cells autologous response was no greater than that achieved with uncultured lymphoma cells. As CD80 expression was higher with IL4 the fact that these cells produced stronger MLRs would be in fitting with the expected result. There did not appear to be a relationship between the number of cells expressing CD80 and the strength of response suggesting that perhaps a small degree of upregulation would be sufficient - if CD80 is the reason for the enhanced MLR.

CD80 expression is directly induced by IL4 and anti-CD40, the lower expression in the IL3/IL10 cells would be expected as one cause of upregulation (IL4) is absent. In fact IL10 has been shown to reduce CD80 in monocytes[54] and IL3 is not known to be an inducer of CD80 expression presumably the only exogenous stimulus in these cells was anti-CD40.

The ability of cells with relatively low CD80 expression (although increased compared to the uncultured cells) to elicit a strong MLR may also be taken as suggesting the role of other adhesion accessory molecules to be important. In particular CD86 (B7.2) and B7.3 were not measured and it may be that some

compensation from these antigens in the low CD80 expressing cells was possible.

The discovery that CD80 expressing melanoma cells were able to act as tumour vaccines in a murine model was the major prompt for this work. The situation is complicated by the fact that the lymphoma cell is acting as antigen presenter - as a malignant cell and as a normal B cell - in its response to cytokine driven CD80 induction.

The fact that autologous MLRs occurred which were stronger with the cultured cells particularly the IL4 cultured cells is encouraging, as this suggests it may be feasible to culture a patients lymphoma *in vitro* - using IL4/anti-CD40 (as this not only produces stronger MLRs than IL3/IL10 group but also leads to a greater growth rate with the generation of more cells), take these cells and inject them into the patient and they then could activate the patients own T cells to generate an immune response.

Whilst the experiments described show a greater MLR occurs with cultured cells whether the reaction generates cytotoxic lymphocytes is currently unknown and it has not been proven that the presence of CD80 is what is responsible for the enhancement of the MLR. Specific blocking experiments using anti-CD80 or CTLA4 Ig would be required to assess the contribution of upregulating the CD80 system to enhancing the MLR.

In summary, *in vitro* culture of lymphoma cells using a stromal cell system is able to enhance the immunogenicity of these cells particularly if grown IL4 and upregulate CD80 expression - this is seen with both allogeneic and autologous T lymphocytes. Whether CD80 expression is the cause of the enhanced MLRs remains to be determined.

CHAPTER 8

CALCIUM CHANNEL BLOCKADE - DIHYDROPYRIDINE AND RYANODINE RECEPTOR SYSTEMS

8. CALCIUM CHANNEL BLOCKADE - DIHYDROPYRIDINE AND RYANODINE RECEPTOR SYSTEMS

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8.1 Introduction

The observation that *bcl-2* expression might lead to apoptotic supression and that calcium flux modification might attenuate or enhance this lead to attempts to modify calcium flow to alter cytotoxic drug sensitivity in *bcl-2* producing cells. It became apparent that the ryanodine receptor antagonists dantrolene and azumolene were able to induce cell death directly and studies on the timing and dosing of these agents are described as are detailed comparisons with conventional cytotoxic drugs. Cell line work is presented initially followed by results obtained in primary culture using the IL3/IL10 stromal cell system.

8.2 *Bcl-2* - the regulation of apoptosis and alterations in calcium flux

The realisation that uncontrolled cell division was not the only cause of tumour development, but that failure of cell death could lead to the accumulation of tumour cells and indeed that this might be the predominant mechansim of growth in slow growing tumours has opened the door to new strategies designed at augumenting this process. The process of programmed cell death or apoptosis - originally described by Wyllie et al in 1972 requires nuclear shrinkage[116] with DNA degradation followed by membrane permeability changes.

Bcl-2 - a major anti-apoptotic protein - now considered a member of a large family of such compounds was first found to be upregulated in follicle centre lymphoma - by virtue of the t(14;18) which placed it under the control of the IgH (immunoglobulin heavy chain) enhancers[62]. This translocation - originally thought to be pathognomonic of such lymphomas is now known to occur in benign lymphoid tissue B cells[62]. It is now becoming clear that over expression of *bcl-2* per se is not sufficient for an anti-apoptotic effect. Heterodimerisation with *bax* is usual - and inhibition of *bax* homodimerisation may be the main mode of its action. In normal lymphocyte development, *bcl-2* expression varies - being high in B cell precursors[63,117] falling at the pre-B cell level before rising again with further maturation. Upregulation at normal stages of low expression may allow malignant cells a significant growth advantage. *Bcl-2* expressed by B cells is a long lived protein with a $t^1/_2$ of about 10hrs[117] - low grade lymphomas lacking t(14;18) also produce it e.g. B-CLL[118] - and it may be

upregulated by cytokines e.g. IL4[50]. Apoptosis induced by DNA damage can also be inhibited by *bcl-2*[119,120], whilst other members of the family e.g. *bcl-2*[121] - a *bcl-2* homologue, may act as the dominant apoptotic suppression protein, as may viral proteins e.g. EBV-BHRF-1[122] which may take on the role of *bcl-2* when this is not expressed. *Bcl-2* action is downstream of DNA repair, or nucleotide pools[62]. It is a 25-26 kd protein present in the ER, nuclear envelope and outer mitochondrial membrane.

Calcium flux changes between the various intracellular compartments appear to occur during apoptosis and may be modulated by *bcl-2*. In a hematopoietic cell line which is IL3 dependent[123], transfection with *bcl-2* can reduce apoptosis. Without *bcl-2*, Ca^{2+} enters the cytosol but with it no change occurs. There appears to be two major stores of calcium: (1) Non-Mitochrondrial - especially[123] - endoplasmic reticulum (ER) (high affinity, low capacity) and (2) Mitochrondrial.

Thapsigargin which inhibits Ca^{2+} ATPases leads to discharge of calcium from non-mitochrondrial stores leading to an initial cytosolic rise followed by a gradual fall in calcium. In an IL3 dependent cell line, on IL3 withdrawal thapsigargin mobilised less calcium in *bcl-2* lacking cells. Mitochondrial calcium levels rose in the absence of *bcl-2*. Calcium ionophores e.g. ionomycin lead to a cytosolic rise in calcium and suppression of apoptosis in *bcl-2* lacking cells[123]. It seems that in the absence of *bcl-2* there is a shift of Ca^{2+} from the ER and cytosol to the mitochrondria. Alteration of Ca^{2+} flux may be able to attenuate or enhance

apoptosis. The effects of *bcl-2* may not be confined to calcium[2124]. It appears to affect lipid peroxidation and oxygen free radical synthesis and this may represent an important mechanism of action. *Bcl-2* does not act in isolation and interactions with various oncogenes has been seen[125].

C-myc expression leads to mitosis and apoptosis. Apoptosis is mediated by a secondary rise in *p53*, co-operativity between *bcl-2* and *c-myc* leads to suppression of *c-myc* induced apoptosis[125,126] but not in its mitogenic role. Cytokines in addition to *bcl-2* are able to suppress *c-myc* induced apoptosis[72]. Insulin like growth factor (IGF-1) and platelet derived growth factor (PDGF) have also been shown to do this as well[72].

Bcl-2 and Chemotherapy

In a leukaemia cell line[120] *bcl-2* protects against the cytotoxicity of dexamethasone, methotrexate, cisplatin, cytarabine and etoposide. Interestingly, its protective effect against vincristine is incomplete. Other studies suggest that the relative protection afforded by *bcl-2* varies from one drug to another[127] - with some e.g. 4 hydroperoxycyclophosphamide or etoposide it is mild, with others e.g. cladribine, methotrexate - it may amount to a one logarithim difference. In acute myeloid leukaemia[128] a high level of *bcl-2* predicts a poor response to chemotherapy.

8.3 Calcium channel blocking drugs - the dihydropyridine and hydantoin derivatives

The use of calcium channel blocking drugs as therapeutic tools is well established in clinical practice. Most of these drugs block the inward flux of calcium by attaching themselves to extracellular voltage dependent receptors which are present in smooth muscle lymphocytes and myocardial cells[129]. Their main role is in the treatment of cardiovascular disorders - angina, hypertension and the prevention of secondary vasospasm following subarachnoid haemorrhage. Nimodipine (Figure 1), a dihydropyridine derivative was used to block extracellular to cytosolic calcium flux.

Dantrolene sodium (Figure 2), a hydantoin derivative[130] or its water soluble analogue azumolene[131,132] (Figure 3) have been used in the treatment of malignant hyperthermia. They bind to the ryanodine receptor in skeletal muscle[133] and prevent the release of calcium from the sarcoplasmic reticulum. Dantrolene binding sites have been found in neural tissue[133-135] and also in murine lymphocytes[136].

Azumolene is structurally related to dantrolene but has a bromo group substituted for a nitro group in the C1 benzene ring and also (see below) an azo substitution for carbon in the oxazoyl ring.

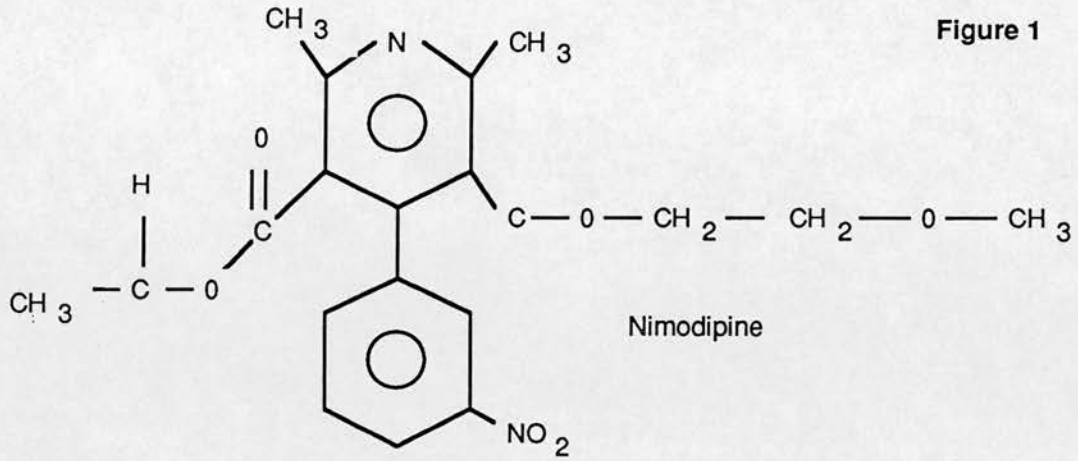


Figure 1

Nimodipine

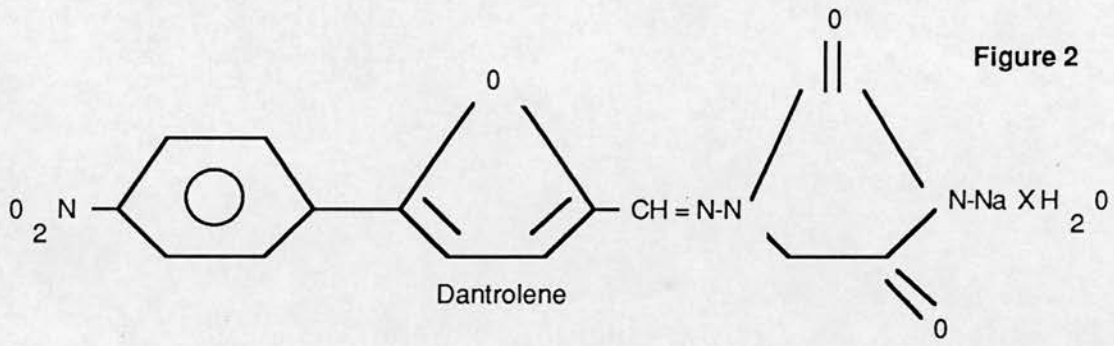


Figure 2

Dantrolene

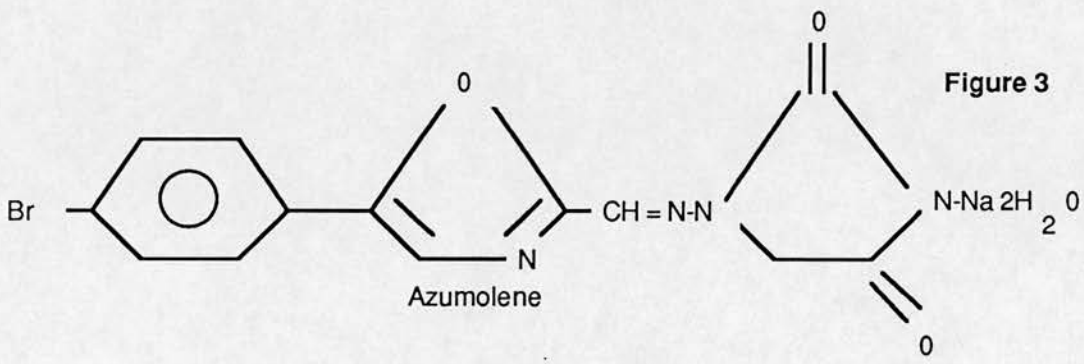


Figure 3

Azumolene

Azumolene is approximately 30 times more soluble than dantrolene[131] and *in vivo* studies in malignant hyperthermia susceptible pigs suggest it to be equipotent[131] as do studies in rat soleus muscle[137]. However, *in vivo* - dantrolene appears three times more potent than azumolene[138] as far as skeletal muscle

relaxation is concerned. It has also been noted to affect Ca^{2+} release induced by doxorubicin from skeletal and cardiac muscle[139]. This effect was most prominent in skeletal muscle and azumolene appeared to bind the sarcoplasmic reticulum at sites distinct from calcium binding sites and inhibit calcium release by competing with doxorubicin at the drug receptor. It seemed as though the binding were not directly to ryanodine binding sites, but that competition for the doxorubicin binding site occurred which reduced the ability of the drug to cause Ca^{2+} release from the sarcoplasmic reticulum.

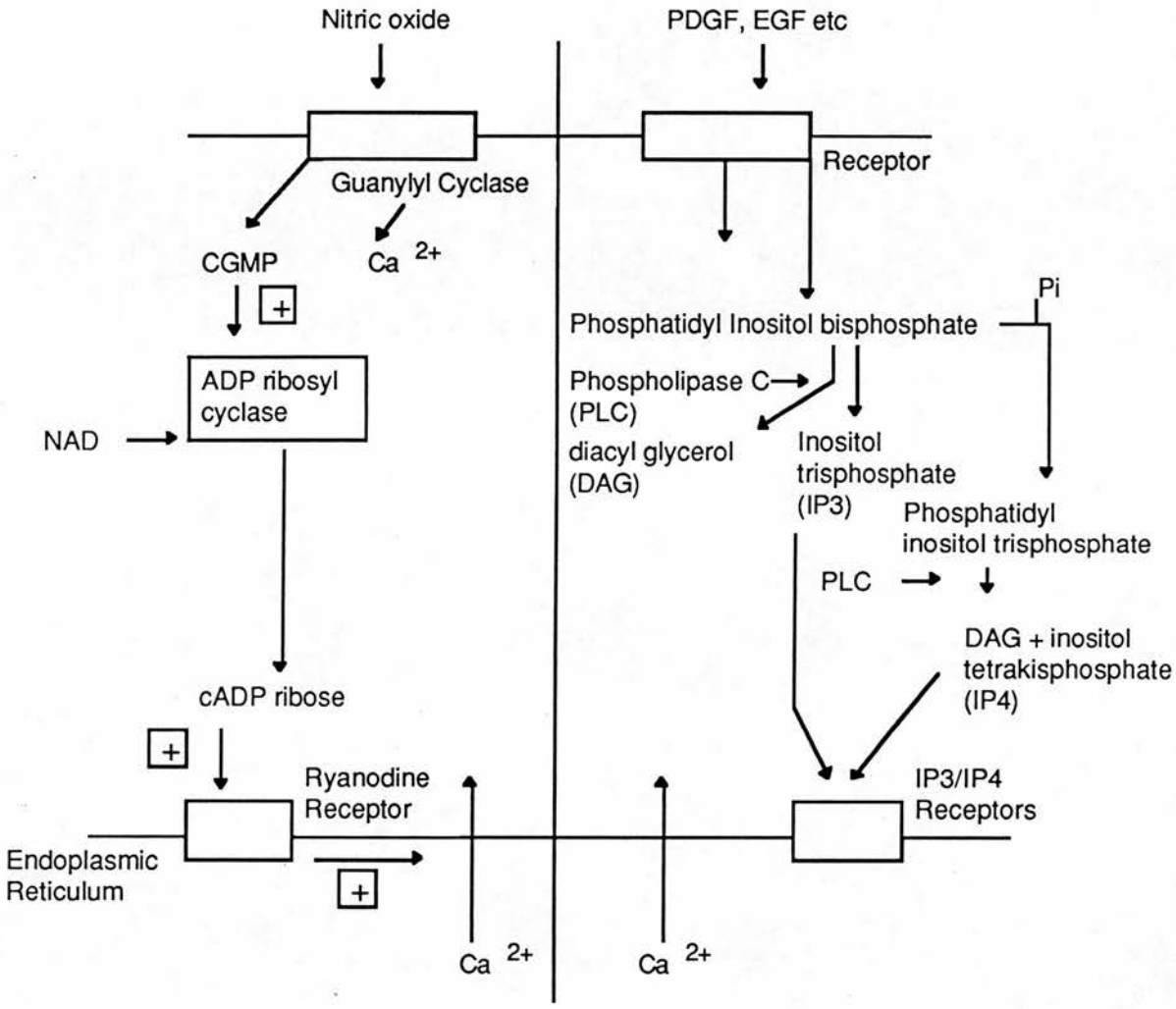
The natural agonist at the ryanodine receptor appears to be cyclic ADP-ribose (cADPR)[140] which with members of the inositol polyphosphate family[141,142] control the influx of calcium from the endoplasmic reticulum to the cytosol. The potent immunosuppressant tacrolimus (FK506) binds to a protein which alters ryanodine receptor function[143] and blocks calcium release in an asymmetrical fashion[144]. Interference with cADPR synthesis from NAD by cladribine (2CdA) a profoundly lymphotoxic drug leads to cell death by apoptosis[12]. As the partitioning of calcium between the endoplasmic reticulum and the cytosol seems important in preventing cell death and this is affected by *bcl-2* - modulation may either be expected to induce death directly or at least enhance the ability of cytotoxics to do so. This provided the rationale for investigation of nimodipine, dantrolene and azumolene firstly in 3 lymphoma cell lines with varying expressions of *bcl-2* (Vallois - very high; $DOHH_2$ - high; Karpas -

Nil) and then in primary cultured low grade B cell lymphoma using the IL3/IL10 anti-CD40 stromal cell system.

In addition, as it had been suggested that a neuroblastoma cell line grew unaffected by dantrolene[145] and that this blocked the growth suppressing effects of oestrogens and anti-oestrogens by interfering with second messenger calcium systems a titration study to see if neuroblastoma cells responded differently to lymphoma in the presence of dantrolene was carried out.

8.4 Schematic diagram of calcium signalling (Figure 4)

Figure 4: Calcium Signalling - the cADP ribose and Inositol Polyphosphate Receptor Systems



Complete blockade of either the ryanodine or inositol polyphosphate systems can lead to compensation by the unaffected one[146].

8.5 Materials and Methods

8.5.1 *Cell Lines*

a) The $DOHH_2$ cell line (t 8;14;18) kindly provided by P. Kluin, University of Leiden[147] - a transformed follicle centre lymphoma expressing *bcl-2*.

b) The Vallois cell line[147] - a lymphoma cell expresing large amounts of *bcl-2* - with a complex translocation.

c) The Karpas 1106 cell line[148] - a lymphoma cell line with a complex translocation involving 18q21.3 but lacking *bcl-2* rearrangement and expression.

d) The SKNSH cell line - a neuroblastoma cell line expressing *bcl-2*.

All cell lines were grown in RPMI base medium (described in Chapter 2). The $DOHH_2$, Karpas and Vallois cell lines were grown with a starting concentration of 10^6/ml in Falcon 25ml flasks -10ml per flask. The neuroblastoma cell line was grown at an initial concentration of 10^5/ml. These grew adherently to the flask and required EDTA 2mmol/L to free them.

Cell counts were manually carried out using a hemocytometer with viability being assessed by 0.2% trypan blue exclusion.

8.5.2 *Drug Preparation*

a) Nimodipine

RMM 418 - supplied by Bayer A.G. Yellow crystalline powder laevorotatory form responsible for calcium channel blockade. Light sensitive - in presence of UV light $t^1/_2$ = 16hrs; in daylight $t^1/_2$ = 56hrs.

In vivo - Action is seen at 2.5×10^{-7}M (0.25µmol/L).

- Insoluble in H_20 - soluble in ethanol.

Range studied 0.01 - 1µmol/L

Solution preparation:- 1mmol/L = 418mg/L
per 10ml = 4.18mg
per 20ml = 8.24mg

Therefore for 10ml solution: 10µl/10ml = 1µmol/L
1µl/10ml = 0.1µmol/L
0.1µl/10ml = 0.01µmol/L

b) Dantrolene Sodium

RMM 399 (hydrated) orange powder; slightly soluble in H_20. Therapeutic range 5-10µmol/L. Solubility in physiological pH aqueous solution = 37µmol/L - maximum. For higher solubility either use organic solvent - 1, 2, 3 propanetriol (glycerol) - requires sonication or adjust pH with NaOH.

Preparation using propanetriol 25mg/10ml

1ml/10ml = 600µmol/L

0.1ml/10ml = 60µmol/L

10µl/10ml = 6µmol/L

1µl/10ml = 0.6µmol/L

Dantrolene in Mannitol/NaOH/H_20 - used for primary culture with:

50mg dantrolene +

100µl of 1 molar NaOH +

75ml H_20 (distilled) +

5g/Mannitol

Therefore: 50mg/75ml = 0.67g/L

Mixture was sonicated for 2 minutes and stored in the dark for 48hrs maximum.

RMM = 399 = stock solution = 1.675×10^{-3}m/L

1.675 millmol/L

1.675µmol/L

For 40µmol/L = 0.4µmol/10ml.

$\frac{0.4}{1675}$ L = 2.38×10^{-4}L ~ 240µl solution

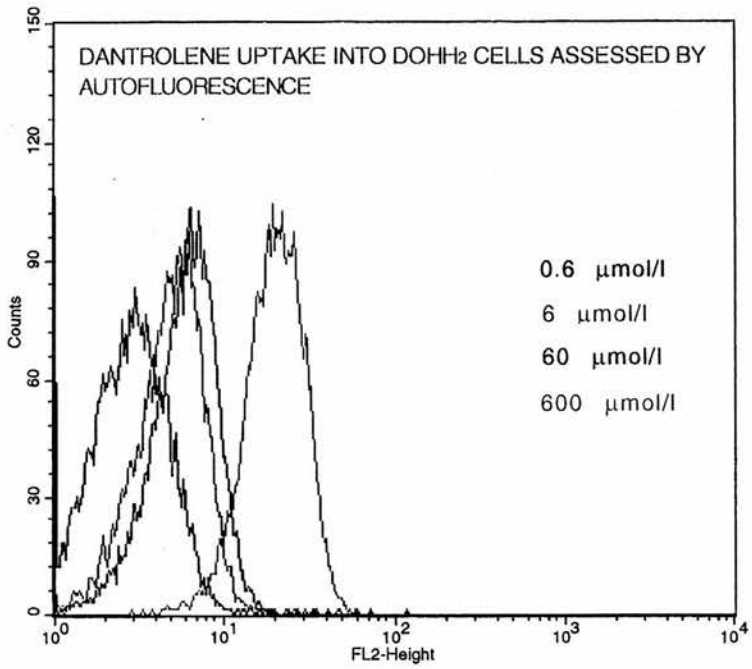
Therefore solutions of:

60µmol/L	=	360µl/10ml
40µmol/L	=	240µl/10ml
20µmol/L	=	120µl/10ml
10µmol/L	=	60µl/10ml
1µmol/L	=	6µl/10ml

Dantrolene autofluorescence in the FL-2 band

In glial cells autofluorescence suggested uptake[134]. This was used to confirm uptake of dantrolene.

Figure 5: Dantrolene uptake into $DOHH_2$ cells assessed by autofluorescence.



c) Azumolene

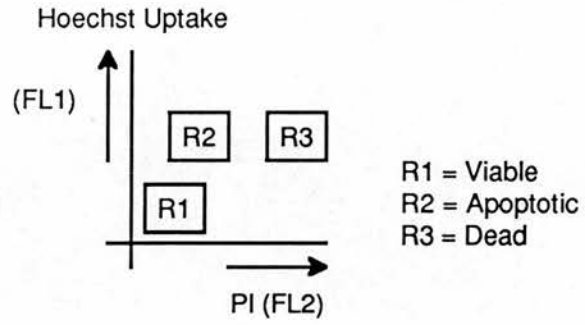
RMM hydrated 453. Isotonic solution 10mg/ml; water soluble white powder. At 9.06mg/ml = 20mmol/L (stock solution).

For 10ml solution =	= 100μmol/L (needs 1μmol)
	= 50μl solution
50μmol/L	= 25μl/10ml
25μmol/L	= 12.5μl/10ml
10μmol/L	= 6.5μl/10ml

8.5.3 *Assessment of Apoptosis*

This was carried out using flow cytometry. Cells were suspended in medium at a concentration of no more than 10^6/ml. To 1ml of suspension were added 100μl of propidium iodide (PI) (50μg/ml) and 100μl of Hoechst 33342 (10μg/ml). The cells were analysed after incubation for 2 minutes at room temperature. A dual laser FACStar was used. The primary laser was set at 488nm to excite PI and the secondary laser at the UV wavelength (351.1 - 363.8) to excite the Hoechst. Both signals were logarithmically amplified with PI fluorescence being detected above 600nm (FL-2 band) and Hoechst fluorescence between 390-460nm (FL-1 band). Results were displayed as a quadrantic plot with Hoechst uptake along the X axis and PI uptake along the Y (see Figure 6).

Figure 6: Quadrantic plot showing uptake of Hoechst dye and Propidium Iodide of live, apoptotic and dead cells



Those cells which took up neither dye were deemed to be viable, those which took up both were deemed to be dead[149,150].

As Hoechst influx is time dependent, all analyses were carried out in the first 5 minutes as eventually all live cells would take up Hoechst. In primary culture, a cruder method of assessment was used by looking at the left shoulder of a PI histogram. This represented the degraded DNA. The higher the percentage in this region compared to the total the greater the number of apoptotic cells. This was compared to untreated controls.

8.5.4 *Viable Cell Count*

In all experiments described, the viable cell count is the total count multiplied by the viability as calculated by assessing 0.1% trypan blue exclusion.

8.5.5 *BrdU Staining*

To ascertain whether azumolene had any effect on cell cycle the DOHH2 cells were stored with BrdU for varying periods of time - 24, 48 and 72hrs prior to fixing and analysis using FACS.

Cells were treated with 10µmol/L BrdU incubated in RPMI base for the allotted time period and then fixed in 70% ethanol at 4°C.

A pellet was prepared and the cells washed twice in PBS. The cells were resuspended in 2M hydrochloric acid and left for 30 minutes at room temperature.

The mixture was then centrifuged and washed in PBS X2 and PBS-T (PBS + 0.1% bovine serum albumin + 0.2% Tween 20 pH 7.4) and stained with 50µl anti-BrdU antibody (Sera-Lab) for 20 minutes at room temperature followed by washing 2X in PBS-T.

2nd layer staining using FITC rabbit anti-mouse $F(ab)_2$ fragments (Dako) 50µl at $^1/_{10}$ dilution for 20 minutes at room temperature. The cells were washed in PBS and treated with 100µl ribonuclease (1mg/ml; Sigma) for 15 minutes at room temperature. 300µl PBS and 100µl propidium iodide. 50µg/ml were added and left for 10 minutes. Analysis was carried out by flow cytometry - with gating out of debris using forward and side scatter.

8.6 Results and Statistical Analysis

All results quoted are the mean of experiments which were carried out in a minimum of two times.

Statistical analyses were carried out by balanced multiple analysis of variance (Minitab, Ver 10, Ohio, USA). The residuals from this were tested to confirm normality of distribution. This was found to be the case ($p>0.1$) except in the case of nimodipine alone (Figure 9) ($p = 0.01$). However, as there was clearly no effect with nimodipine no non parametric analysis was performed.

8.6.1 *Prolonged culture with continuous dantrolene and/or nimodipine in the DOHH$_2$ (Figures 7, 8, 9)*

The total viable count was analysed at 72 and 156hrs. Cell death was progressive, with a fall in cell numbers apparent at 72hrs. Dantrolene was studied at 3 concentrations - 60, 30 and 15μmol/L and nimodipine at 1, 0.5 and 0.25μmol/L. Balanced analysis of variance for time and treatment revealed a significant effect ($p<0.01$). No advantage over single agent dantrolene was seen ($p = 0.859$). Only the higher concentration (D - 60μmol/L) actually reduced cell numbers compared to the starting amounts, whilst in the lower concentration a growth retarding effect was seen. No evidence of activity for nimodipine alone was observed ($p = 0.645$) (Figure 9). Eventually the maximum effects of dantrolene matched those of the combination, but it took longer. Flow cytometry showed the mode of death to be necrotic, with minimal induction of apoptosis.

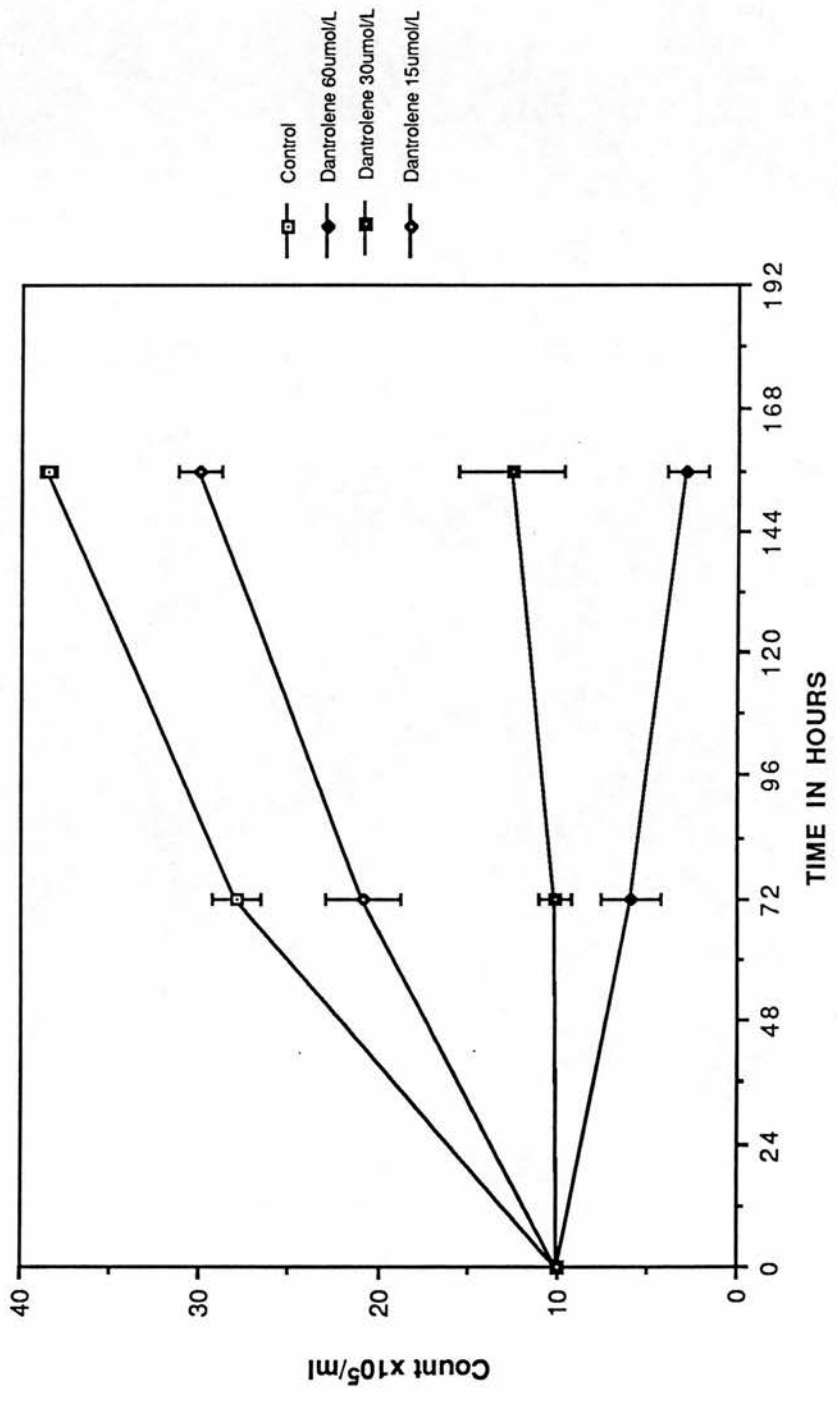


Figure 7: Effect of different concentrations of dantrolene in the DOHH2 cell line

Figure 8: Combined blockade with increasing concentrations of dantrolene and nimodipine in the DOHH2 cell line

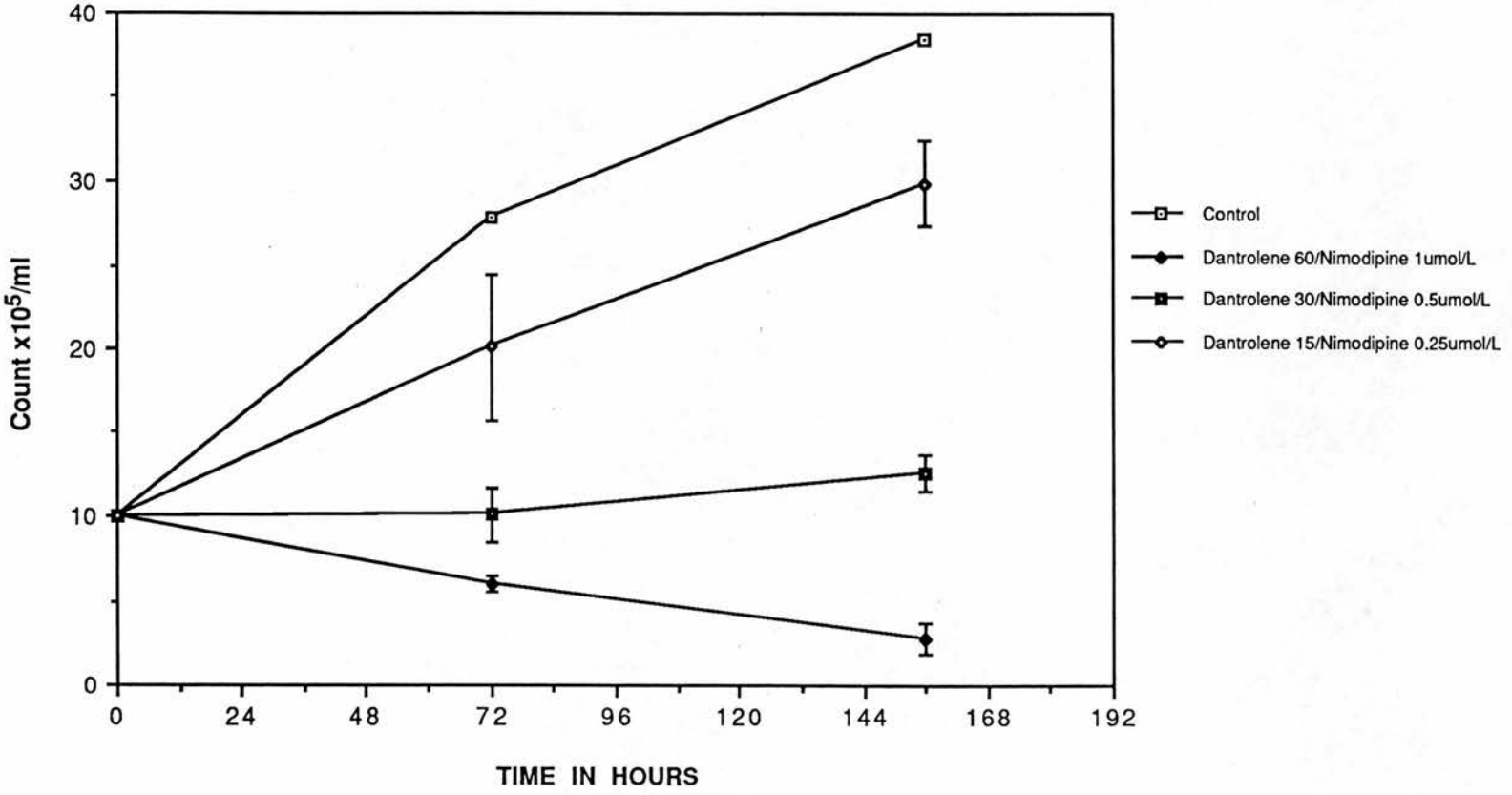
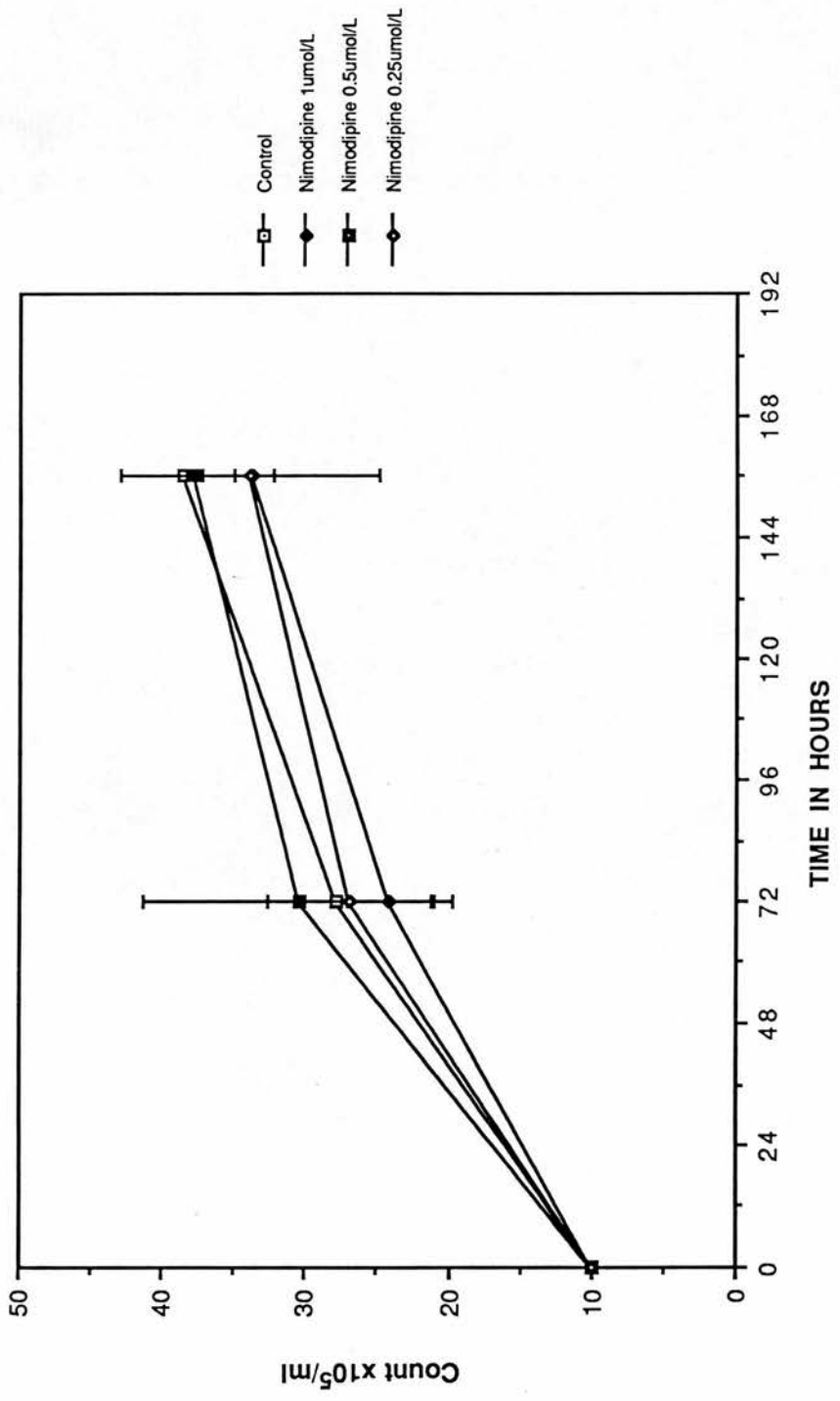


Figure 9: The effect of increasing concentrations of dantrolene and nimodipine in the DOHH2 cell line



4.6.2 *Single agent dantrolene in the Karpas and Vallois, $DOHH_2$ and SKNSH cell lines 168hrs incubation (Figures 10-13)*

Following the demonstration of a significant cytotoxic effect of dantrolene in the $DOHH_2$ cell line the 2 cell lines with differing levels of *bcl-2* expression as assessed by flow cytometry were used to assess dantrolene activity. The cells were exposed to varying concentrations of dantrolene for 168hrs and then the viable cell count calculated. The results confirmed the Vallois line with the highest level of *bcl-2* production to be the most sensitive, the Karpas line which produced no *bcl-2* was less sensitive (Karpas vs Vallois $p = 0.054$), however it was also growing more slowly and the $DOHH_2$ which grew the quickest was actually least sensitive (Karpas vs $DOHH_2$ $p = 0.5$, $DOHH_2$ vs Vallois $p = 0.09$). This suggests that if *bcl-2* is responsible for the difference, no correlation between the level of *bcl-2* production and the degree of resistance to dantrolene can be inferred. It is reassuring that the Karpas line which was growing significantly more slowly was more sensitive than the $DOHH_2$ suggesting that cell growth rate was not the determining factor.

<u>The SKNSH cell line</u>

Cells were incubated for 168hrs with dantrolene at: 20, 40 and 60μmol/L prior to assessing the viable cell line. The line expressed *bcl-2* strongly (see Figure 14 - FACS analysis). The line seemed much less sensitive to dantrolene than the lymphoma lines.

Figure 10: Karpas - 168hr exposure to Dantrolene: cell count at 168hrs

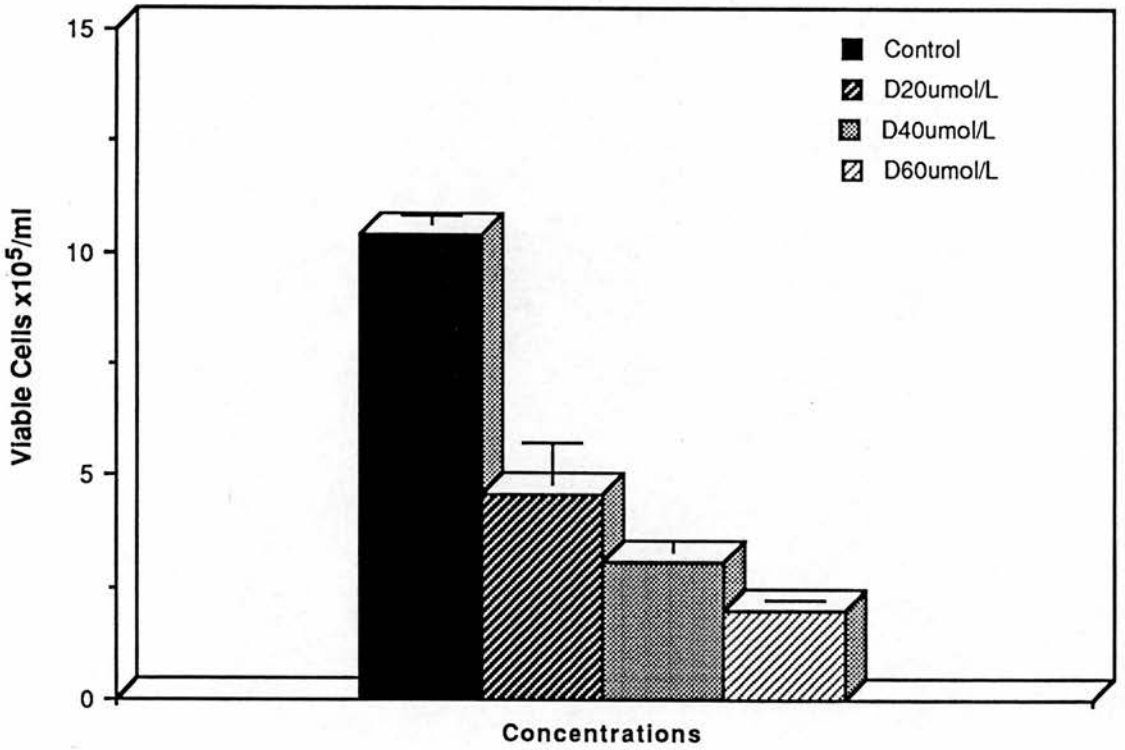


Figure 11: Vallois - 168hr exposure to Dantrolene: cell count at 168hrs

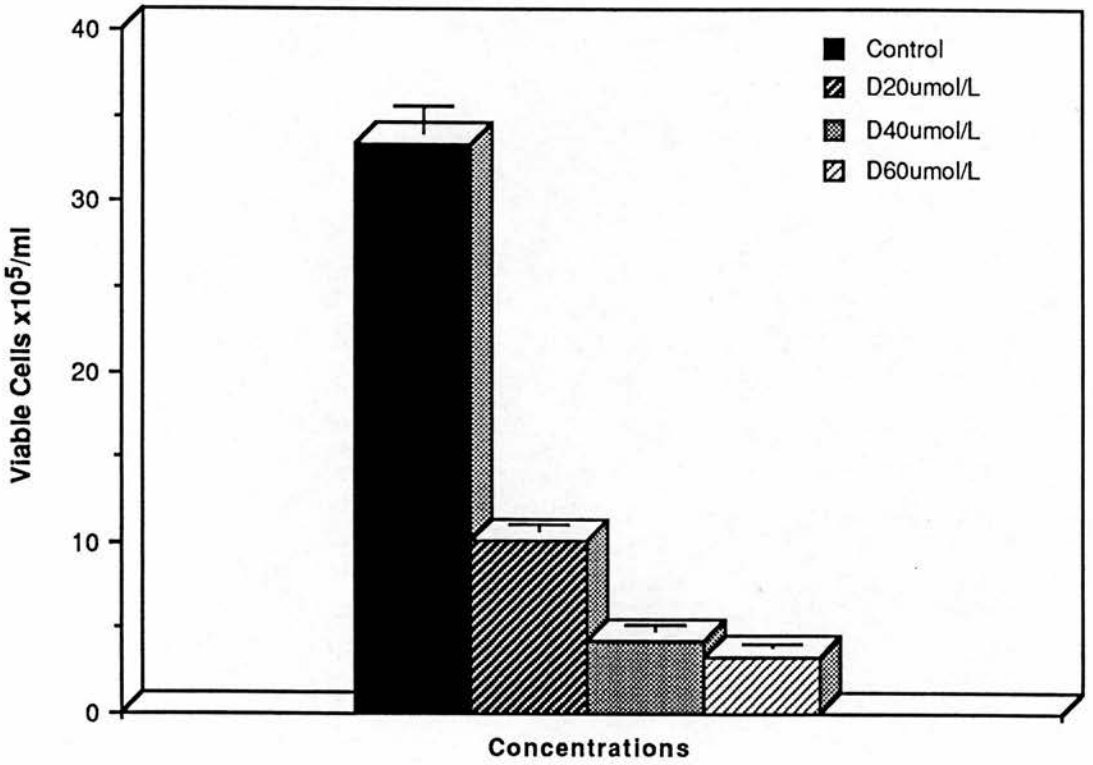


Figure 12: DOHH2 168hr exposure to Dantrolene: cell count at 168hrs

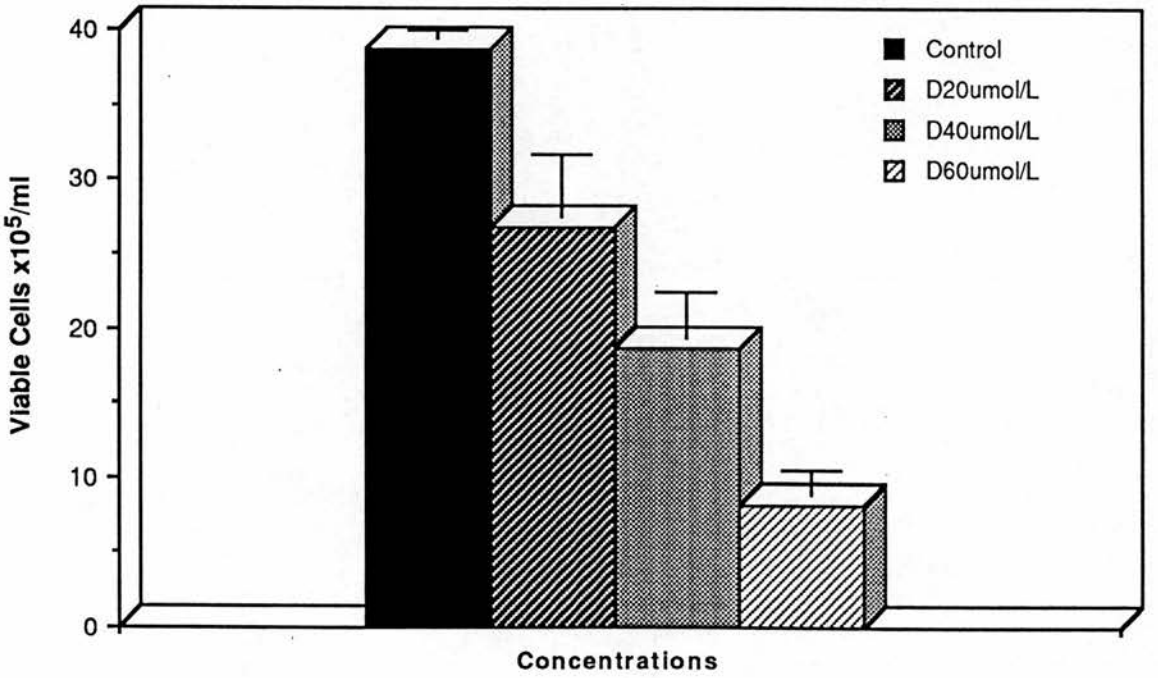


Figure 13: SKNSH - 168hr exposure: cell count at 168hrs to Dantrolene

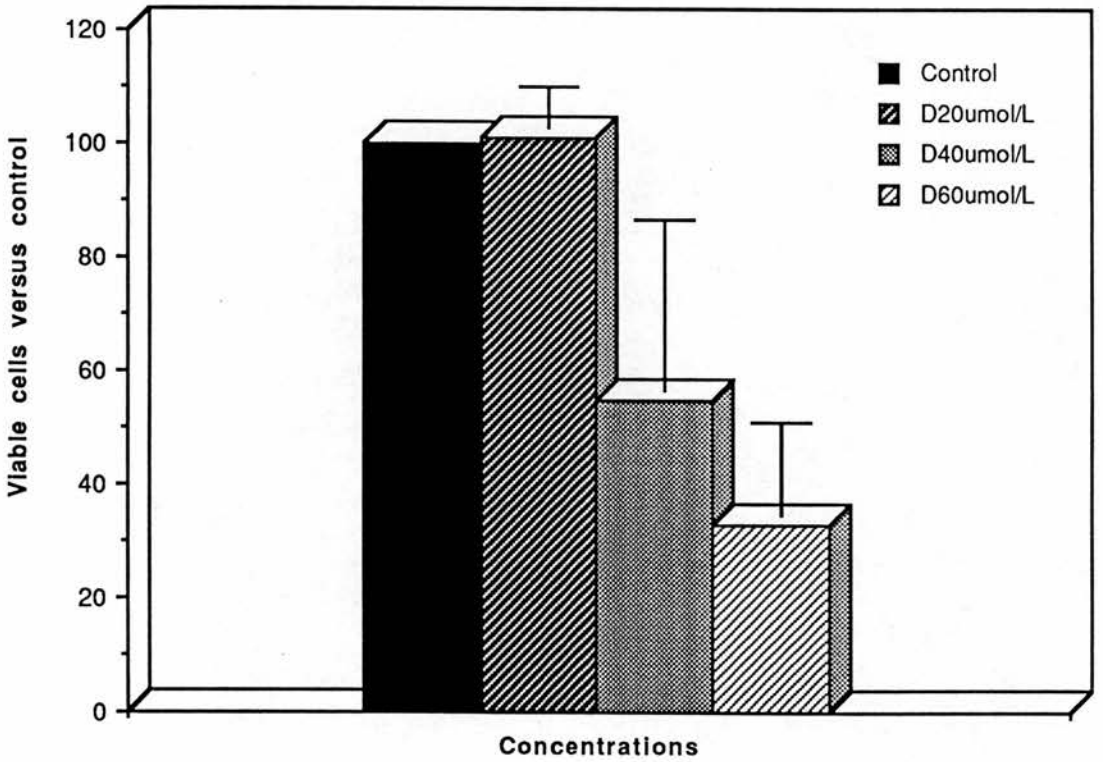
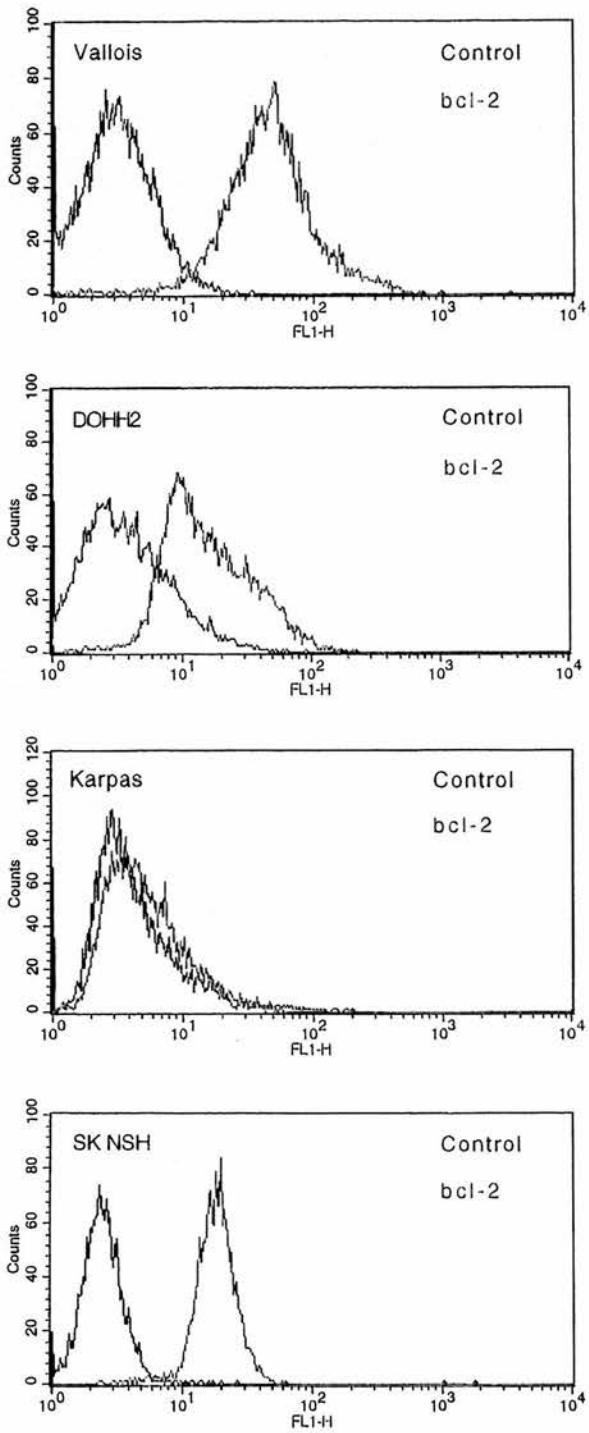


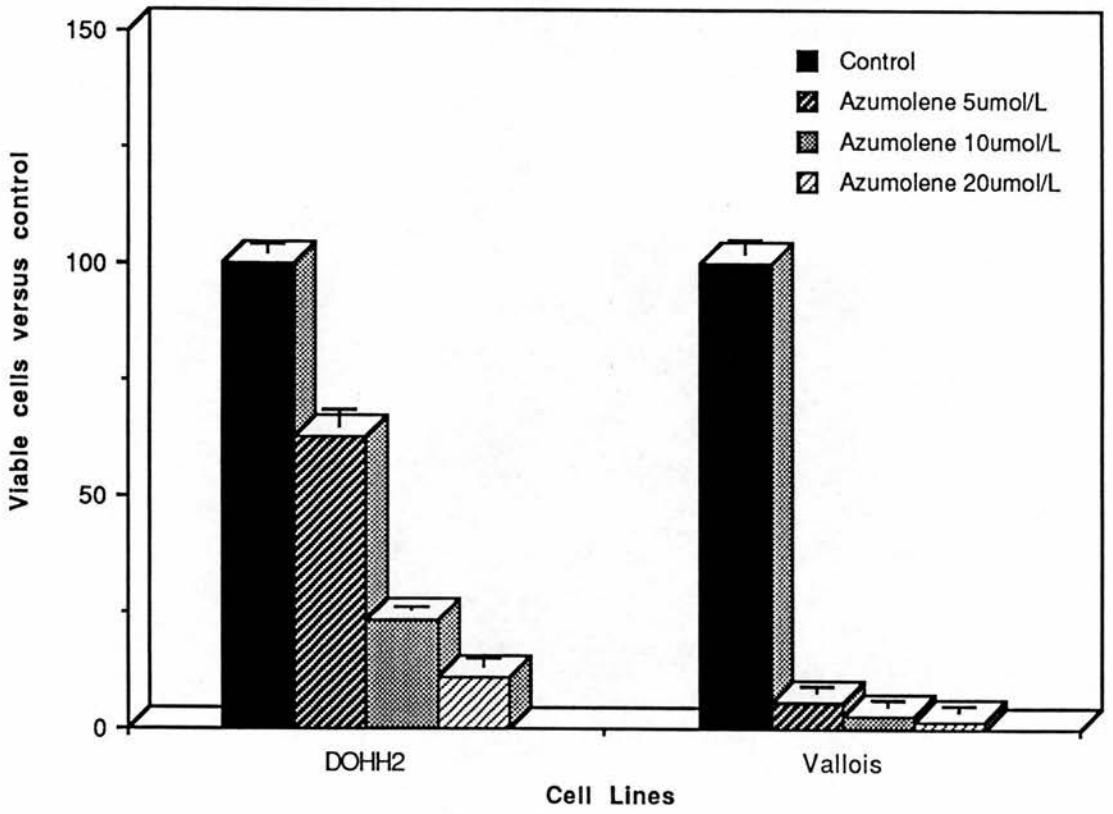
Figure 14: FACS histograms showing *bcl-2* expression in the Vallois, $DOHH_2$, Karpas and SKNSH cell lines



8.6.3 *Azumolene Titration*

Azumolene was titrated for effect in the DOHH2, Karpas and Vallois cell lines. As previous studies had suggested it to be equivalent as far as muscle relaxation was concerned a range of 5-20μmol/L was selected. In the 3 cell lines a dose response was seen with azumolene 10μmol/L ~ dantrolene60μmol/L. The results in the 3 cell lines are shown compared to dantrolene (Figure 15).

Figure 15: 72hrs Azumolene - cell count at 168hrs



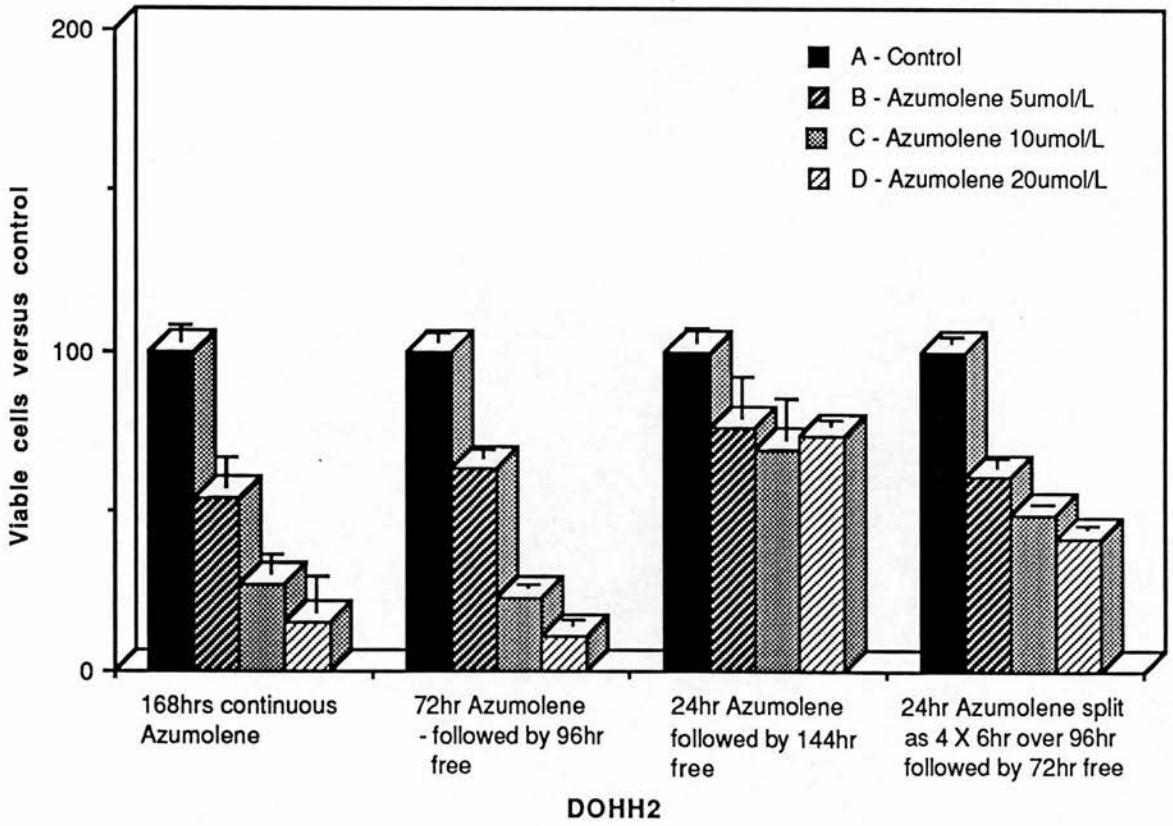
8.6.4 *Scheduling of Azumolene (Figures 16 and 17)*

Since azumolene had been found to be as efficacious, although significantly more potent than dantrolene, further studies were carred out to see if its effect on cell lines were schedule dependent, and if the effect of a short incubation period were as effective as a longer one. The following were investigated.

(1) 168hrs continuous azumolene - cell count at 168hrs (Figure 14).
(2) 72hrs azumolene - cell count at 168hrs.
(3) 24hr azumolene - cell count at 168hrs.
(4) 24hr azumolene divided in four 6 hourly exposures at 24hr intervals - with cell count at 168hrs.

The experiments were carried out in the $DOHH_2$ and Vallois cell lines. For the 24 and 72hr experiments samples were washed in RPMI base medium and washed twice before resuspension in RPMI base for culture. This was carried out at the end of each 6 hourly exposure during the fractionated azumolene.

Figure 16: Analysis at 168hrs versus control in DOHH2 cell line



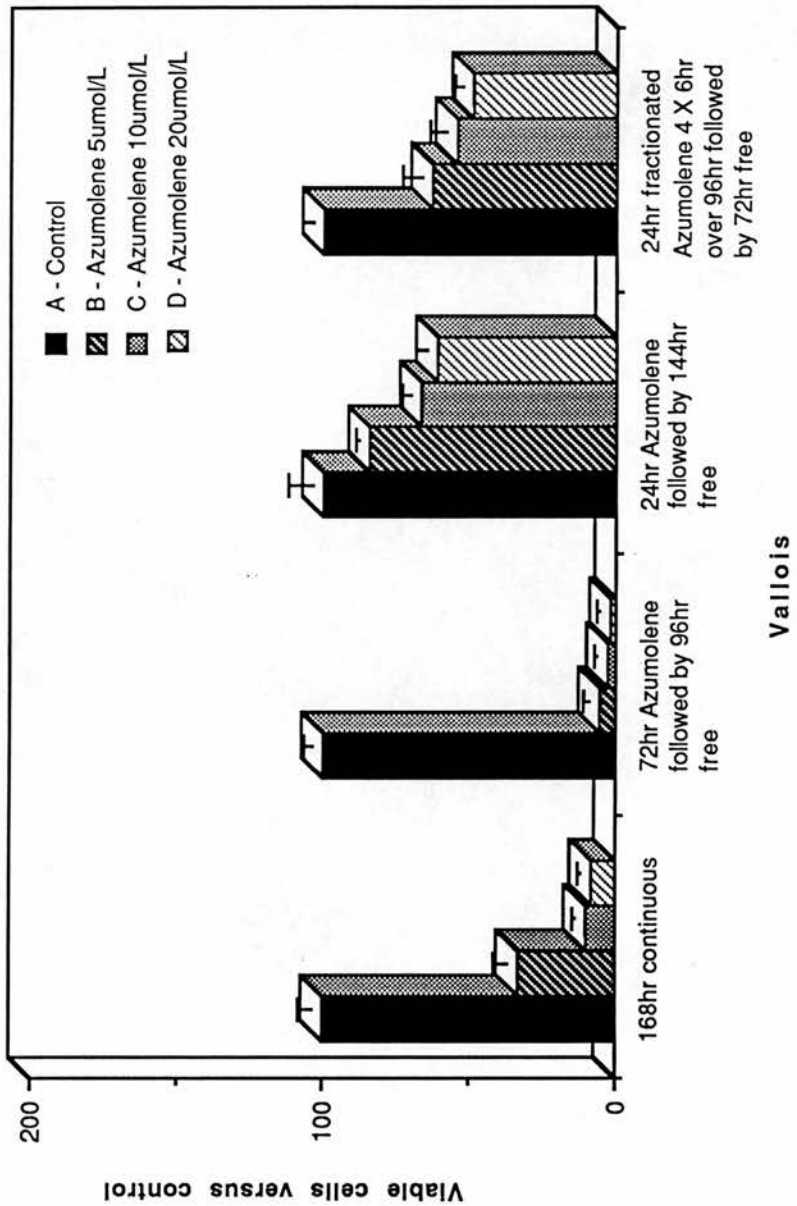
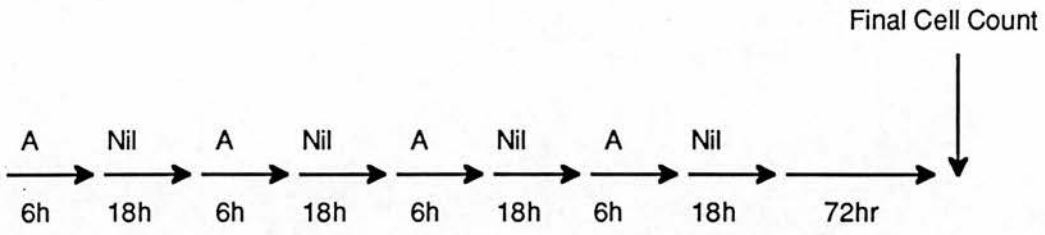


Figure 17: Analysis at 168hrs control in Vallois cell line
A - Control
B - Azumolene 5umol/L
C - Azumolene 10umol/L
D - Azumolene 20umol/L
Viable cells versus control
200
100
0
168hr continuous
72hr Azumolene followed by 96hr free
24hr Azumolene followed by 144hr free
24hr fractionated Azumolene 4 X 6hr over 96hr followed by 72hr free
Vallois

Figure 18: Diagram of 4 X 6 hourly fractionated Azumolene scheme



A = Azumolene

Continuous exposure to Azumolene over 168hrs lead to the best results. 72hrs showed intermediate results (72hrs vs 168hrs $p = 0.08$) whilst 24hrs continuous exposure followed by 144hrs recovery in Azumolene free medium appeared the weakest. Fractionating the dose in to four 6 hourly exposures appeared to enhance the effect (24hrs vs 4 x 6hrs $p<0.001$) but it still remained inferior to the 72 or 168hrs exposure groups. The fact that 24hr exposure given as 4 X 6 hourly exposures in 96hrs was more effective than 24hrs in one go suggests that intracellular levels may be maintained. In experiments carried out on the primary lymphoma cell line specimens continuous exposure was used for fear of disrupting the growing cells from the monolayer with repeated washes.

8.6.5 *BrdU analysis and apoptosis assessment - using Flow Cytometry - (see Figures 19 and 20)*

There was no evidence that cell cycling was arrested by azumolene at any time or at any concentration studied suggesting

that any cytotoxic effect of azumolene is non cell cycle specific. This was the case in the $DOHH_2$ and Vallois cell lines. Repeated sampling at 24 hourly intervals for evidence of apoptosis showed evidence of this particularly at the higher concentrations (Azumolene 10 and 20μmol/L). It was seen in the $DOHH_2$ cell line but not in the Vallois line and as this expressed *bcl-2* to a greater degree than the $DOHH_2$ line - this is further evidence that azumolene did not exert its cytotoxic effect via *bcl-2* interruption.

Figure 19: DOHH2 cell line: analysis of apoptosis using flow cytometry over a 72hr period

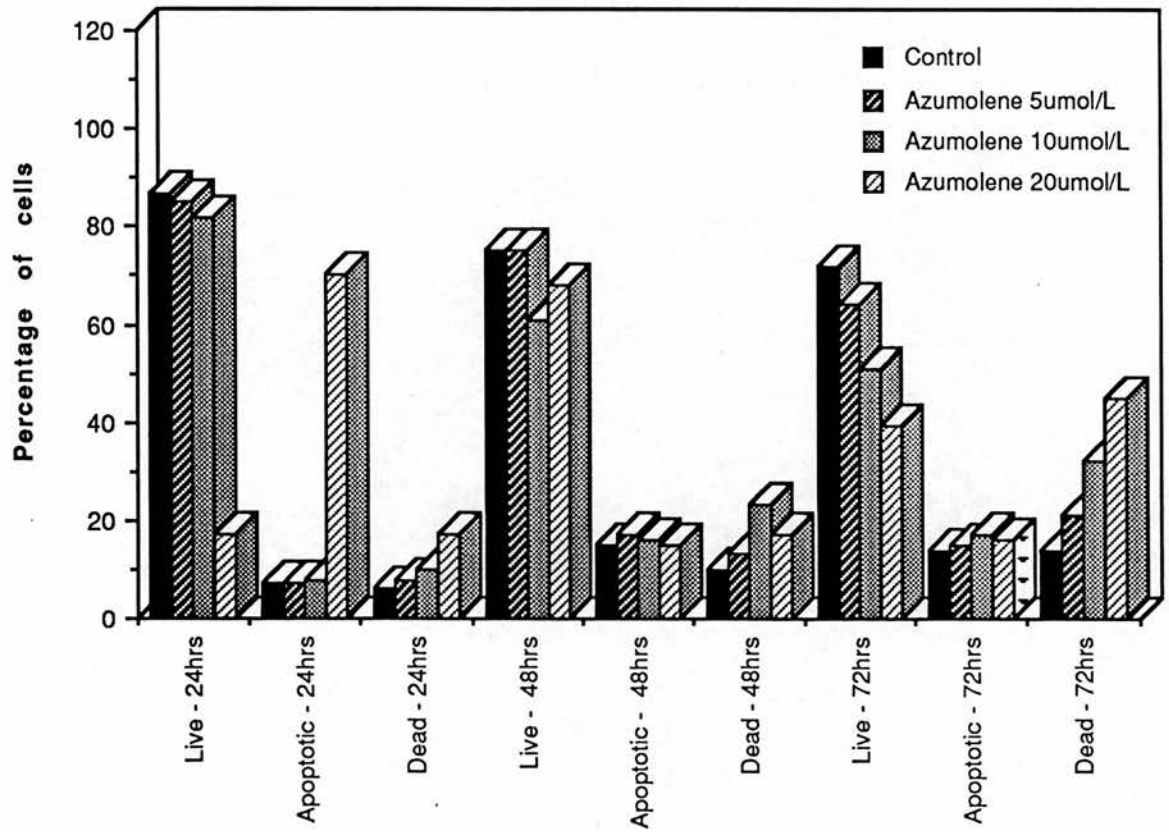


Figure 20: Vallols cell line: analysis of apoptosis using flow cytometry over a 72hr period

8.6.6 *Ryanodine receptor antagonists in primary lymphoma culture (see Figures 21a + b and 22)*

The experiments were carried out using the stromal cell system previously described. 24 wells were set up. The difficulty in changing the medium without disrupting the lymphoma cells and fibroblasts meant only continuous exposure to dantrolene or azumolene could be investigated. The lymphoma cells were cultured on the monolayer for 3-5 days to ensure they were established prior to the addition of the drugs which were then applied for 120hrs prior to analysis. 4 wells were treated per drug and cells harvested at the end of the experiment fro the 4 wells, centrifuged and resuspended in exactly 1ml PBSA. Counting was carried out using a haemocytometer, viability was assessed with trypan blue as before. 2 concentrations of dantrolene were used - 60μmol/L and 30μmol/L in the 1st experiment. In the comparison with azumolene 60μmol/L of dantrolene was compared to 6μmol/L of azumolene. 60μmol/L and 30μmol/L of dantrolene appeared equivalent, and 60μmol/L dantrolene appeared similar to 6μmol/L azumolene.

There did not appear to be any relationship between the sensitivity of a particular culture to the ryanodine receptor antagonists and the level of *bcl-2* expression.

Figure 21a

The effect of dantrolene at 2 concentrations in 5 low grade lymphoma cultures.

Histology	FCL L211	SLL L304	FCL L503	FCL L1003	FCL L1203
Growth Fraction (%)	7	3	6	6	10
Day 1					
Day 10	7	2	4	6	11
Bcl-2 Expression % versus negative control	97	0	9	47	92
Day 1					
Day 10	36	93	35	80	6

The effect of dantrolene versus that of azumolene in 3 lymphoma cultures.

Histology	FCL L214	FCL L802	FCL L903
Growth Fraction %			
Day 1	7	10	7
Day 10	7	N/A	6
Bcl-2 Expression % versus negative control			
Day 1	97	4	92
Day 10	36	N/A	89

Figure 21b: The effect of dantrolene at 2 concentrations in 5 low grade lymphoma cultures

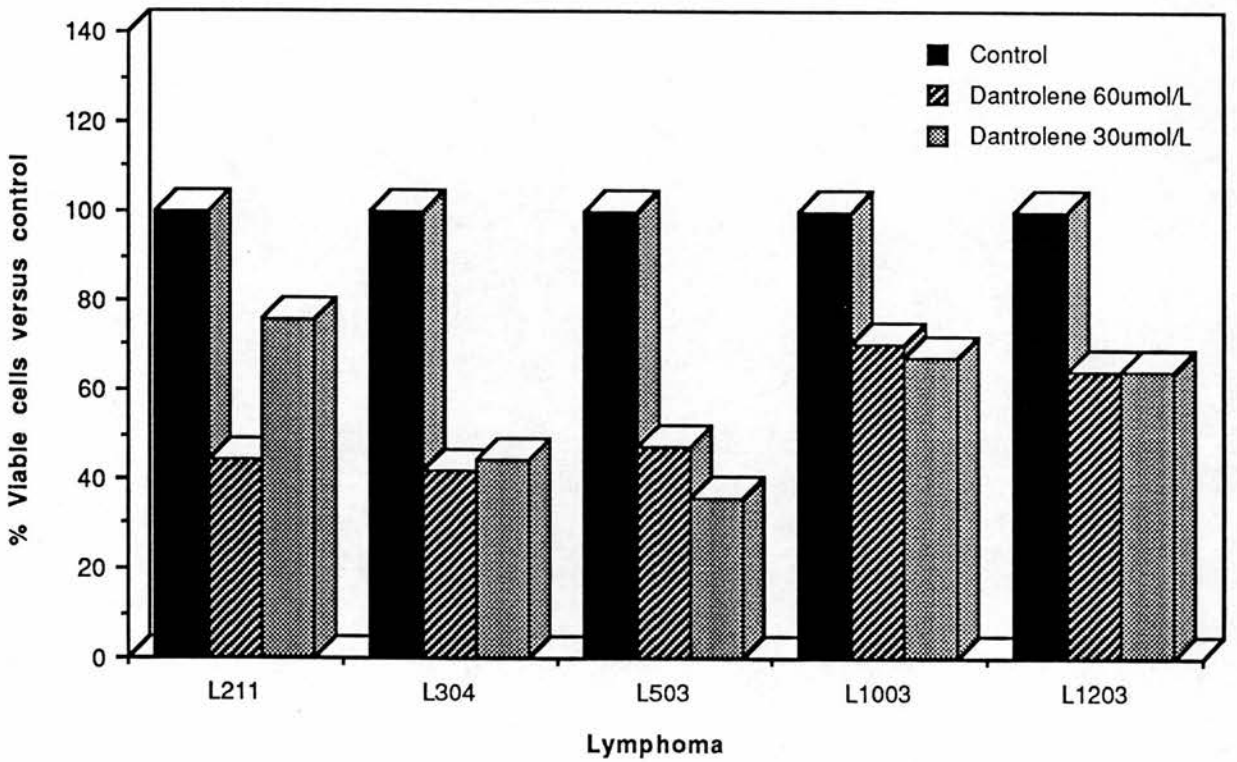
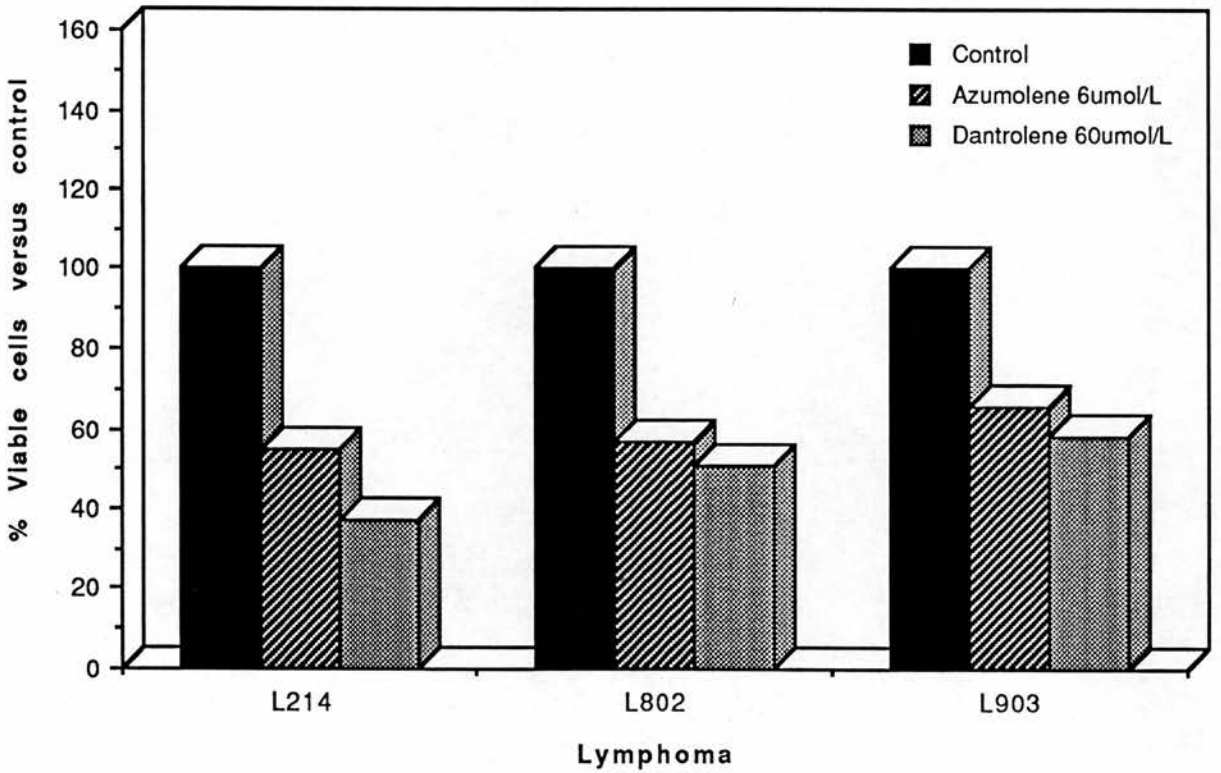


Figure 22: The effect of Dantrolene versus that of Azumolene in 3 lymphoma cultures



In primary culture 6μmol/L azumolene was used. As cell line work suggested 10μmol/L to be equivalent to 60μmol/L dantrolene, the inferior results obtained here with 6μmol/L azumolene are not surprising. There appeared to be no correlation between growth rate or *bcl-2* expression and senstivity to these agents. Compared to cell lines, the primary cultures appeared relatively resistant to both these drugs.

Assessment of Apoptosis

In L214, L802, L903 there was no evidence from PI histograms that death was via apoptosis in the treated cells.

8.7 Discussion

The experiments described, suggest that dantrolene and azumolene have cytotoxic activity in various lymphoid cell lines and more importantly in primary lymphoma culture. Dantrolene appears less potent, although maximal efficacy of the two appears the same. The initial assumption that deregulation of *bcl-2* action is responsible for this effect is not strongly supported by the evidence presented. Although the most strongly expressing *bcl-2* cell line was most sensitive to the ryanodine receptor antagonists, there was little difference between the $DOHH_2$ and the Karpas cell lines which produced some and no *bcl-2* respectively, and no relationship could be seen in the primary lymphoma cultures. Fractionation of dose of azumolene appeared more effective than a single exposure in the $DOHH_2$ cell line suggesting that there may be some schedule dependency. The concentrations of dantrolene required for its effects seen here were much higher

than studies into its actions on muscle relaxant with effects being maximal in the 10μmol/L range[119]. *In vitro* work has used concentrations of up to 300μmol/L[133]. Suppresion of intracellular calcium mobilisation needed to stimulate gluconeogenesis in rat hepatocytes has been achieved using 60μmol/L of dantrolene[151]. Very high concentrations have been given to humans with doses likely to produce plasma levels of 40-50μmol/L - without ill effect[152]. High concentrations administered to beagle dogs using lyophilised dantrolene/mannitol and NaOH confirmed a 54% inhibition of skeletal muscle contraction in doses producing levels of 55-60μmol/L with no effect on heart or blood pressure[153]. Azumolene, on the other hand produced significant effect at or slightly above those levels established as being equivalent to dantrolene 10μmol/L[154] as far as skeletal muscle relaxation is concerned and this suggested that it have a higher therapeutic index.

Nimodipine appeared a comparatively disappointing agent. It has little activity alone and combination with dantrolene was no better than dantrolene alone. This may be because most cytosolic calcium changes depend on repartitioning between endoplasmic reticulum and cytosol, rather than receiving calcium from the extracellular space[134]. A similar calcium channel blocker - verapamil has been reported to cause inhibition of B cell proliferation *in vitro* and induce clinical reponses in B cell CLL which might be due to tumour necrosis factor (TNF), release inhibition[155]. In addition, there is some evidence that both dantrolene and azumolene may antagonise dihydropyridine

receptors as well as ryanodine ones[156] thus reducing the additional role of nimodipine.

The timing of cell death suggests a novel action for these drugs as compared to conventional cytotoxics with minimal effects being seen in the first 48 hours. Whilst a cytotoxic action may be postulated for concentrations over 40mmol/L dantrolene (>10μmol/L azumolene) a cytostatic action occurs at lower doses in cell line studies. In primary culture there seems little difference between concentrations of 30 or 60μmol/L.

A cytostatic action may be useful in slow growing tumours. The most appropriate schedule for investigation appears to be azumolene administered daily by bolus injection or short infusion in view of evidence suggesting enhancement of effect by fractionation of dose.

CHAPTER 9

DRUG SENSITIVITY TESTING - IN CELL LINES AND PRIMARY LYMPHOMA CULTURE

9. DRUG SENSITIVITY TESTING - IN CELL LINES AND PRIMARY LYMPHOMA CULTURE

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9.1 Introduction

In vitro drug sensitivity testing is an area of research which still has to overcome significant problems if it is to make a contribution to the management of individual patients as opposed to simply identifying new agents which are worthy of further study. Most *in-vitro* work has been carried out using cell lines, however, these fail to retain certain features of the parent tumour. They may have lost their dependency on local growth factors and may simply represent the most aggressive and rapidly dividing element of a tumour. They are nevertheless, easy to work with and large numbers of cells may be cultured allowing experiments to be repeated numerous times.

For *in-vitro* work to be more representative it is necessary to grow the parent tumour and hope it retains many of its *in vivo* characteristics.

This chapter examines the use of various cytotoxic drugs in the lymphoma cell line ($DOHH_2$) and compares the results with those obtained using the IL3/IL10 stromal cell system for primary lymphoma culture. The dihydropyridine - nimodipine and the ryanodine receptor antagonist dantrolene are combined with cytotoxic drugs to study the effects in this cell line to look for synergy.

Many normal and cancer cells are able to remove toxic compounds by an energy consuming process involving the production of P-glycoprotein. Some cells have this system activated, in others it needs to be induced. The synthesis of P-glycoprotein in cancer cells is a mechanism for acquired drug resistance. It has been observed that verapamil and nifedipine are able to reverse drug resistance by blocking the influx of calcium and antagonising the P-glycoprotein system. Although it has not been reported that nimodipine or dantrolene are able to affect this system, it may be that they do and that this is responsible for at least part of their activity in combination with cytotoxic drugs. It however does not explain the activity they have alone in the absence of cytotoxic drugs. The $DOHH_2$ cell line and some primary lymphoma cultures are surveyed for their ability to produce P-glycoprotein. Finally, the results of the cytotoxic drugs are compared with those of the ryanodine receptor antagonists seen in the previous chapter.

9.2 Materials and Methods

9.2.1 *Dose finding of cytotoxic drugs in the DOHH2 cell line*

Table 1

Drug	Supplier	Formulation	RMM (Relative Molecular Mass)
1. Fludarabine	Schering	Dry powder preservative free - dissolve in H_20 to 25mg/ml	363
2. 4-Hydroperoxy-cyclophosphamide (4HC)	-	Dry powder - dissolve in ethanol - 3mg/ml	300
3. Doxorubicin	Farmitalia	Dry powder with lactose and 0.02% hydroxybenzoate - dissolve H_20 to 2mg/ml	580
4. Etoposide	Bristol Myers Squibb	Dry powder - dissolve in 50% methanol:50% H_20 at 1mg/ml	587
5. Cisplatin	David Bull	Preservative free 1mg/ml	300
6. Cytarabine	David Bull	20mg/ml preservative free	243
7. Vincristine	David Bull	Preservative free 1mg/ml	923
8. Methotrexate	David Bull	Preservative free 2.5mg/ml	454
9. Dexamethasone Phosphate	David Bull	Preservative free 4mg/ml	450

It was decided to investigate several drugs which have been shown to have activity in lymphoma. The drugs were titrated in logarithmically increasing concentrations in an attempt to find the concentration that killed an arbitary 40% of the cells at 48hrs. At the same time the mode of cell death was investigated to see if

apoptosis were induced and then to see if any correlation between this and any interaction with dantrolene existed.

9.2.2 *Cell Line*

The $DOHH_2$ cells were cultured in RPMI base with 10^6/ml; 10ml per flask (Falcon 3013E, 50ml) and incubated at 36°C, 5% $C0_2$ with the chosen concentration of drug for 48hrs (4hrs in the case of cisplatin and 4HC) prior to assessment of apoptosis.

9.2.3 *Primary Culture*

The lymphomas studied were cultured using the IL3/IL10 stromal cell system. Cells were allowed to grow for 3-5 days prior to the addition of an appropriate amount of cytotoxic drug or control to 4 wells of a Falcon 3047 (24 well) plate with initially 10^6 viable cells per well in 2ml of medium. Cells were counted as described previously. In the case of the two Hodgkin's lymphomas studied FACS analysis was carried out to quantify the percentage phenotypes of the cells post treatment as the number of T cells was high and it was possible that all that had happened was that these had been killed, lowering the total cell count.

9.2.4 *Apoptosis*

This was assessed using uptake of Hoechst 33242 and Propidium Iodide as described previously.

9.3 Assessment of P-glycoprotein mediated drug efflux influence by Nimodipine and Dantrolene

The drugs were surveyed using the $DOHH_2$ cell line, primary lymphoma culture cells and peripheral blood lymphocytes which are known to express P-glycoprotein. P-glycoprotein can be inhibited by many different drugs, the immunosuppressive drug cyclosporin was used here for this purpose, the technique being that described by Chaudhary et al[157]. The ability to excrete the fluorescent dye $DIOC_2$ was taken as evidence of a functional P-glycoprotein system. Paralysis by cooling to 4°C, as an alternative to cyclosporin was also used - the two methods producing similar results.

Peripheral blood lymphocytes were prepared by centrifugation on a Ficoll layer. The mononuclear layer was extracted and a pellet prepared.

9.3.1 *Lymphocyte Dyeloading*

The pellet of cells was diluted in 10ml RPMI and 6µl of the dye $DI0C_2$ (100µg/ml) leading to a final concentration of 0.06µg/ml. The tubes were incubated for 25 minutes at 4°C - the low temperature paralysed the efflux system allowing the cells to take up the dye.

9.3.2 *Dye Efflux*

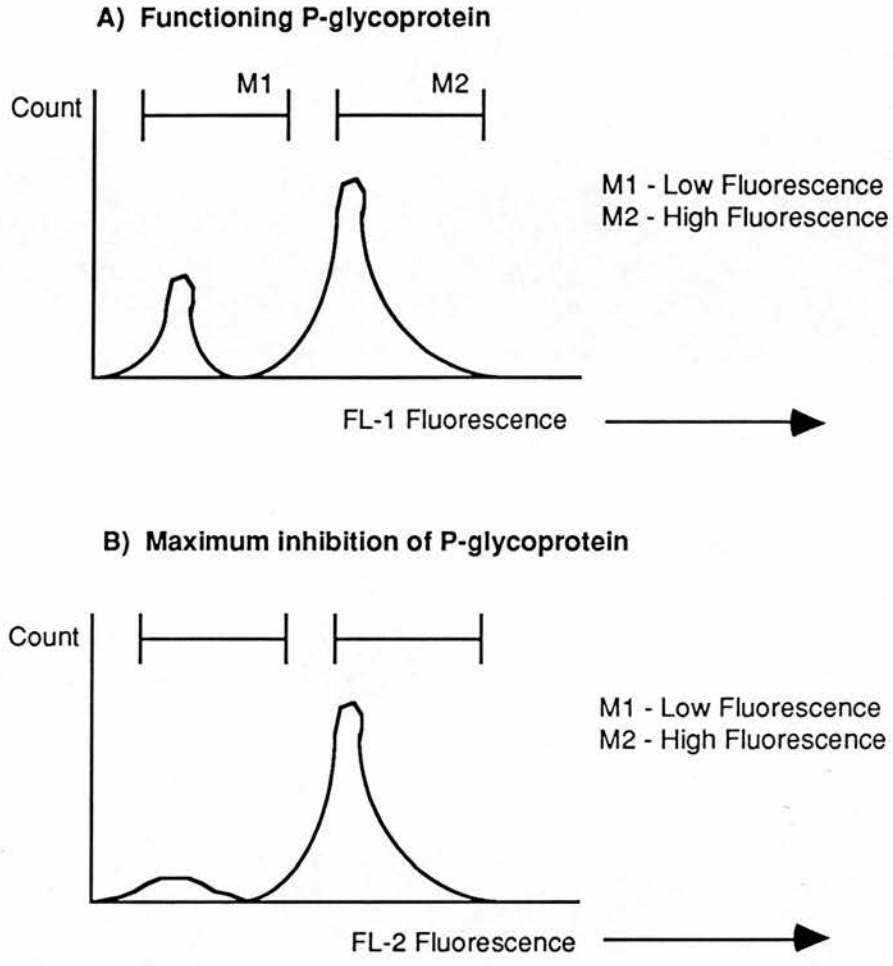
After 25 minutes incubation the tubes were spun at 1600g for 10 minutes and the supernatant discarded. The pellet of cells was resuspended in 10ml of cold RPMI to wash the cells.

The RPMI was discarded and the cells resuspended in RPMI with 10% FCS. 100µl of lymphocyte suspension were put in each of two tubes and kept on ice to use as a control sample with low or no dye efflux. The rest was divided and the drug under investigation added and incubated in a final volume of 5ml of RPMI and 10% FCS at 37°C for 3hrs. A set of duplicate samples containing 2µmol/L of cyclosporin were used as positive controls of P-glycoprotein inhibition.

9.3.3 *FACS Analysis*

After incubation, the samples were spun at 1000g, 10 minutes at 4°C. The lymphocyte pellets were resuspended in ice cold RPMI and washed twice in this before analysis using a FACScan with Lysys II software. Information was displayed on a histogram (see Figure 1). $DIOC_2$ fluorescence occurred in the FL1 band.

Figure 1



- the more cells in the M1 band the more drug effluxed spontaneously. A high M1:M2 ratio suggests a functioning P-glycoprotein system. If cyclosporin or cooling to 4°C is used the M-1 area contains minimal cells suggesting inhibition of the system.

The following concentrations were tested in the cell line and blood lymphocytes and primary lymphoma culture.

Dantrolene (D):	0, 15, 30, 60, 100 μmol/L
Nimodipine (N):	0, 0.25, 0.5 μmol/L
Combined:	D-60; N-1 μmol/L
	D-100; N-1 μmol/L

Controls:	No drug at 4°C (No Efflux)
	2μmol/L cyclosporin (Maximum Inhibition)
	No drug at 37°C (Maximum Efflux)

Primary Lymphoma Culture:	L304 (B cell - Small Lymphocytic Lymphoma)
	L503 (Follicle Centre Lymphoma, Follicular Grade 1)
	L1103 (Follicle Centre Lymphoma, Follicular Grade 3)

5.3.4 *Investigation of pulsed calcium channel blockade using Nimodipine and Dantrolene with cytotoxic drugs*

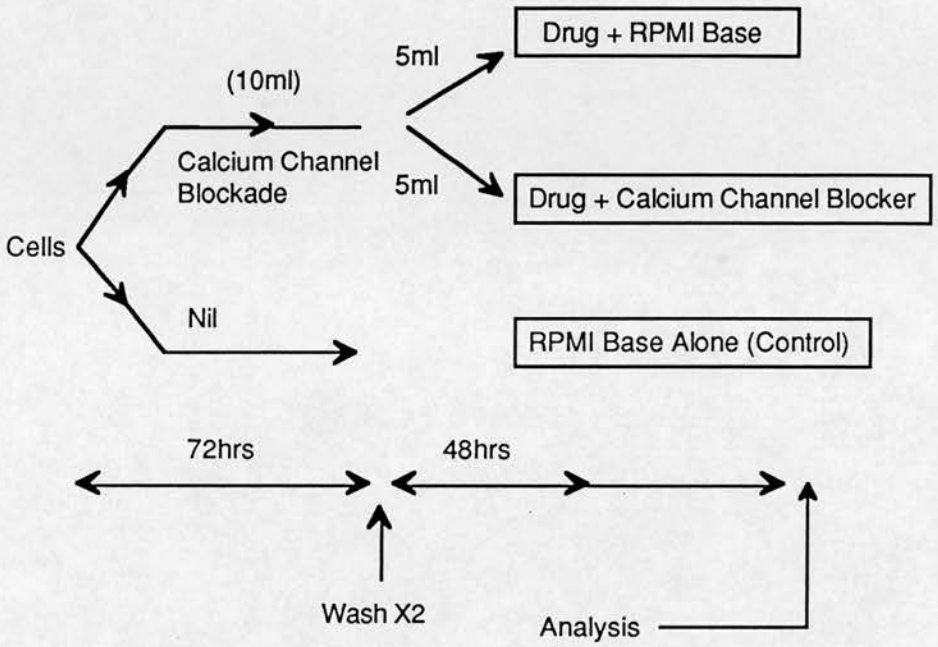
These studies were carried out using the $DOHH_2$ cell line; 10^6 viable cells/ml were taken and grown in flasks (Falcon 3013E); they were incubated with Nimodipine and Dantrolene prior to washing in RPMI base and the addition of cytotoxic drugs with or without calcium channel blockers. Cell counts were obtained using a Coulter Counter. Viability was assessed by 0.2% trypan blue exclusion, apoptosis was assessed using Hoechst 33342 and Propidium Iodide.

(i) <u>72hr pulse calcium channel blockade</u>

72hr pulsed calcium channel blockade using combined nimodipine and dantrolene followed by addition of vincristine (49hr) or cisplatin (4hr) was carried out 48hrs after drug

incubation. The drugs were incubated either in the presence or absence of calcium channel blockers (see Figure 2).

Figure 2



2 concentrations of calcium channel blockers were used:

(i) Dantrolene 60μmol/L + Nimodipine 1μmol/L (DN-1).

(ii) Dantrolene 6μmol/L and Nimodipine 0.3μmol/L (DN-2).

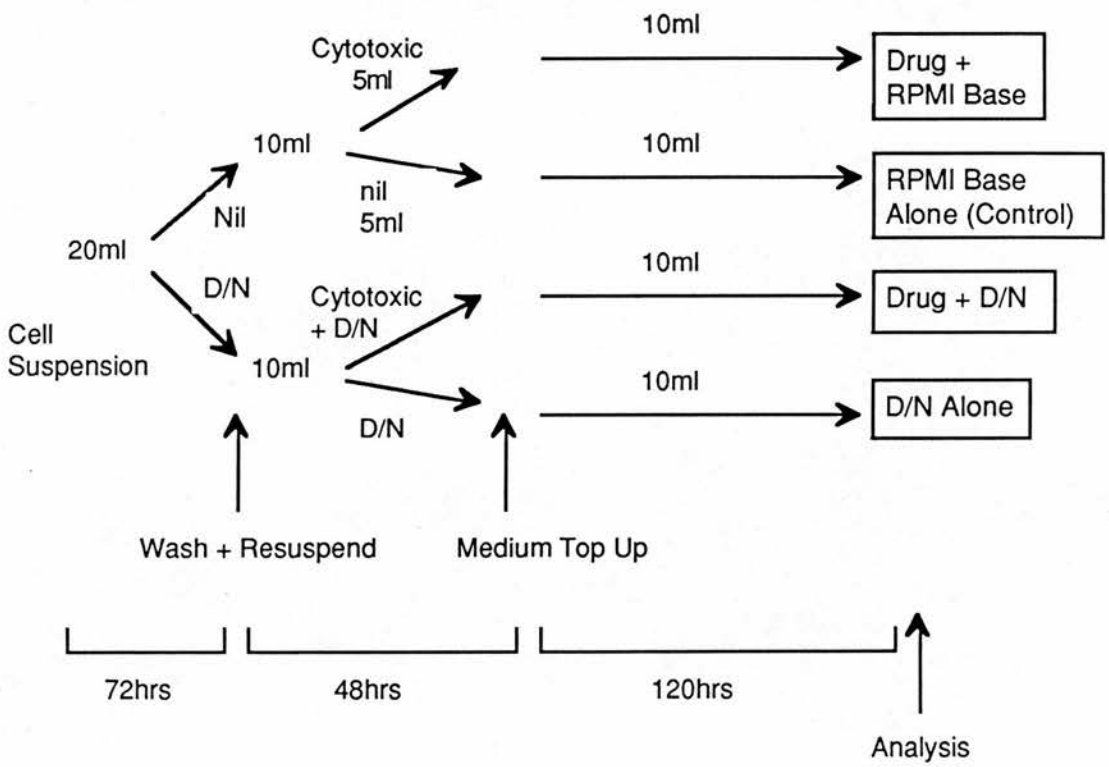
Vincristine at a concentration of 1μmol/L and cisplatin at a concentration of 10μmol/L were used.

(ii) <u>10 Day Experiment to study prolonged pre and post calcium channel blockade (Figure 3)</u>

20ml of $DOHH_2$ cells were taken (10^6/ml) and incubated either with dantrolene and nimodipine (D/N) (6μmol/L and 0.3μmol/L respectively) or in RPMI base alone. At 72hrs both groups were washed 2 times and then a cytotoxic drug

was added for 48hrs either in the presence of dantrolene and nimodipine or not. After this the cells were incubated for a further 120hrs with channel blockers or control solutions and topped up to 10ml, followed by analysis 240hrs after the experiment started.

Figure 3



The Cytotoxics were not washed out after 48hrs, but remained in the solution until analysis.

Drugs: 4HC - 10μmol/L
Cisplatin - 10μmol/L
Cytarabine - 10μmol/L
Doxorubicin - 0.1μmol/L

9.4 Results

9.4.1 *Dose Finding Studies - see Table 2*

Experiments were carried out in duplicate in most cases. The concentrations required to kill >40% of cells at 24hrs or 48hrs is recorded.

Table 2

Drug	Concentrations to kill >40% at 24hrs (μmol/L)	Concentration to kill >40 % at 48hrs (μmol/L)
Fludarabine	1000	100
Doxorubicin	0.1	0.1
Etoposide	10	0.1
Cisplatin	100	10
Cytarabine	1000	10
Vincristine	10	1
Methotrexate	100	10
Dexamethasone	>1000	>1000
4 Hydroperoxy Cyclophosphamide (4HC)	N/A	100

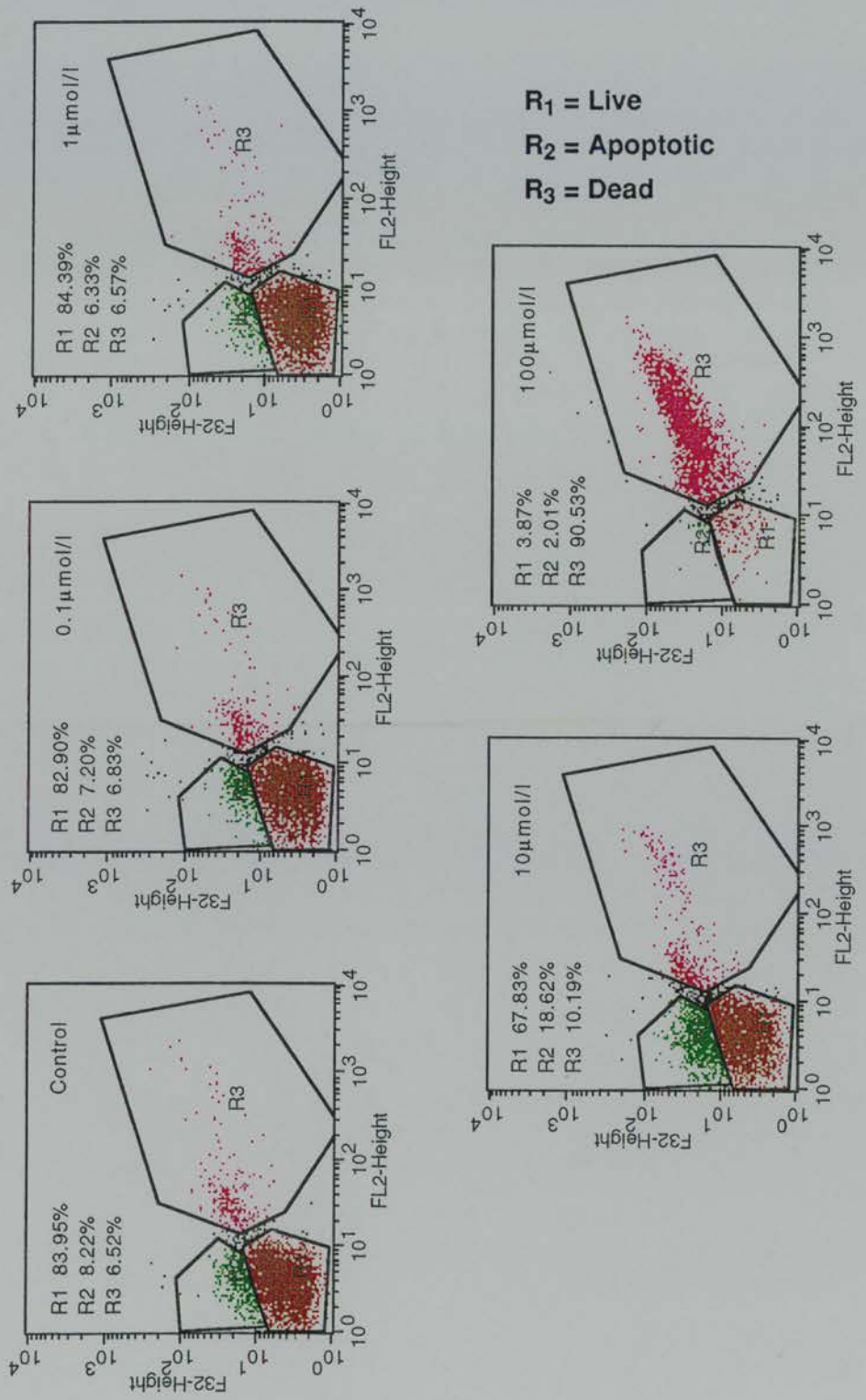
N/A - Not Available

The concentration chosen for future experiments usually lay between the two readings. One exception was fludarabine for

which evidence exists that more prolonged exposure with a lower dose is more effective and so 10μmol/L was chosen.

An example is shown in Figure 4 showing the effect of 4HC on $DOHH_2$ cells.

Figure 4: Assessment of apoptosis in the $DOHH_2$ cell line 48 hours after treatment with different concentrations of 4-hydroperoxycyclophosphamide (4HC)



9.4.2 *Induction of Apoptosis - see Table 3*

Not all drugs had similar profiles and although apoptosis was seen at 24hrs and 48hrs, failure to find this - the cells simply being necrotic, lead to sampling at earlier stages - at 1, 3, 6 and 12hr post drug administration to see if an intervening apoptotic stage had been missed. Despite this, neither doxorubicin nor cytarabine appeared to induce apoptosis. In most cases results were duplicated. The percentage of viable cells 72hrs post chemotherapy versus control is listed (using PI exclusion).

Table 3

Drug	Concentration in μmol/L in $DOHH_2$ cell line	Maximum % of apoptotic cells	% Viable cells
Fludarabine	10	17	60
Doxorubicin	0.1	5	31
Etoposide	10	21	42
Cisplatin	10	27	40
Cytarabine	10	2	52
Vincristine	1	22	30
Methotrexate	10	21	44
Dexamethasone	1000	1	104
4HC	10	20	20

9.4.3 *P-glycoprotein Production.*

Results were expressed as a proportion compared to that seen with 0μmol/L at 4°C - as 4°C is cold enough to inhibit the pump and therefore all dye is thought to be retained. The maximum inhibition with 2μmol/L cyclosporin - corresponds to this as well.

Peripheral blood lymphocytes: maximum inhibition with cyclosporin or control at 4°C were equivalent. At 37°C 75% of cells effluxed the dye suggesting a functioning pump (maximum efflux).

The nimodipine and dantrolene samples all showed a distinct decrease in fluorescent intensity compared to the controls and were equivalent to the maximum efflux control at 37°C. This demonstrated that nimodipine and dantrolene did not functionally inhibit P-glycoprotein.

When $DOHH_2$ cells were used in the assay, all the histogram maps appeared the same as the 100% bright and maximum inhibition controls. Since these demonstrated no efflux this suggested that the cell line did not express P-glycoprotein. In the three primary lymphoma cultures all samples and controls showed no evidence of dye efflux. Thus these also do not appear to have a functional P-glycoprotein system.

In conclusion neither dantrolene nor nimodipine affected P-glycoprotein activity and as neither the $DOHH_2$ cells nor the primary lymphoma cultures appeared to express P-glycoprotein

any synergy with cytotoxic drugs could not be due to P-glycoprotein pump inhibition.

9.4.4 *Combined calcium channel blockade and cytotoxic drugs*

(i) <u>120hr experiment with cisplatin (Pt) and vincristine (VCR) (Figures 5, 6 and 7)</u>

Enhancement of the effect of both drugs is seen. In the case of vincristine pre and post drug treatment with a high concentration of dantrolene and nimodipine appeared most effective, although the effect was modest reducing viable cells from 25% to 9%. With cisplatin a similar effect was seen although here the effect of high or low concentrations of calcium channel blockers pre and post drug and administration made little difference with viable cells reduced from 40% to 24% with the lower concentration of calcium channel blockers, and 40% to 30% with the higher.

Figure 5: **Analysis of cells at 120hrs following treatment with cisplatin and various calcium channel blocking combinations**

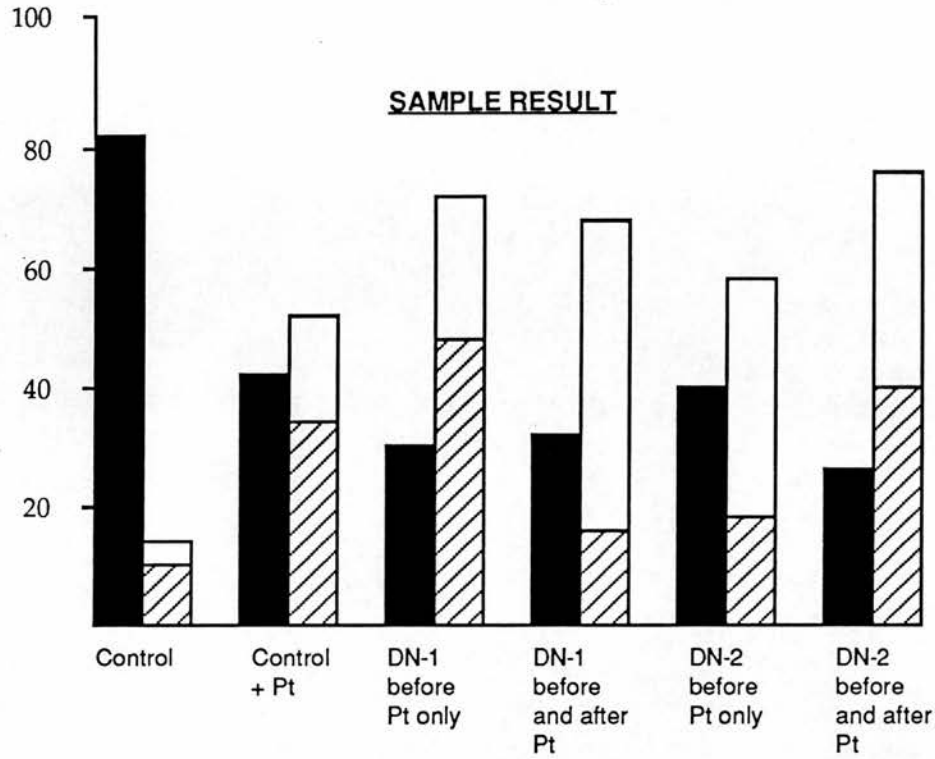


Figure 6: **Analysis of cells at 120hrs following treatment with vincristine and various calcium channel blocking combinations**

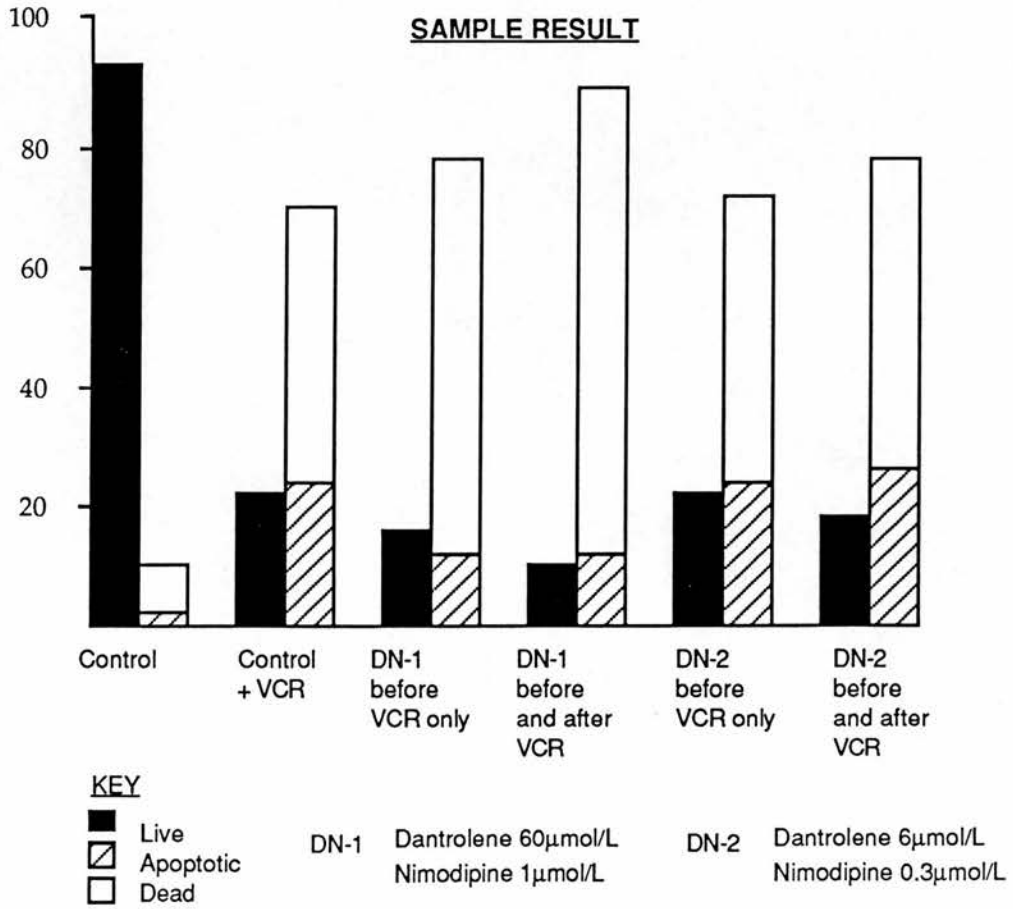
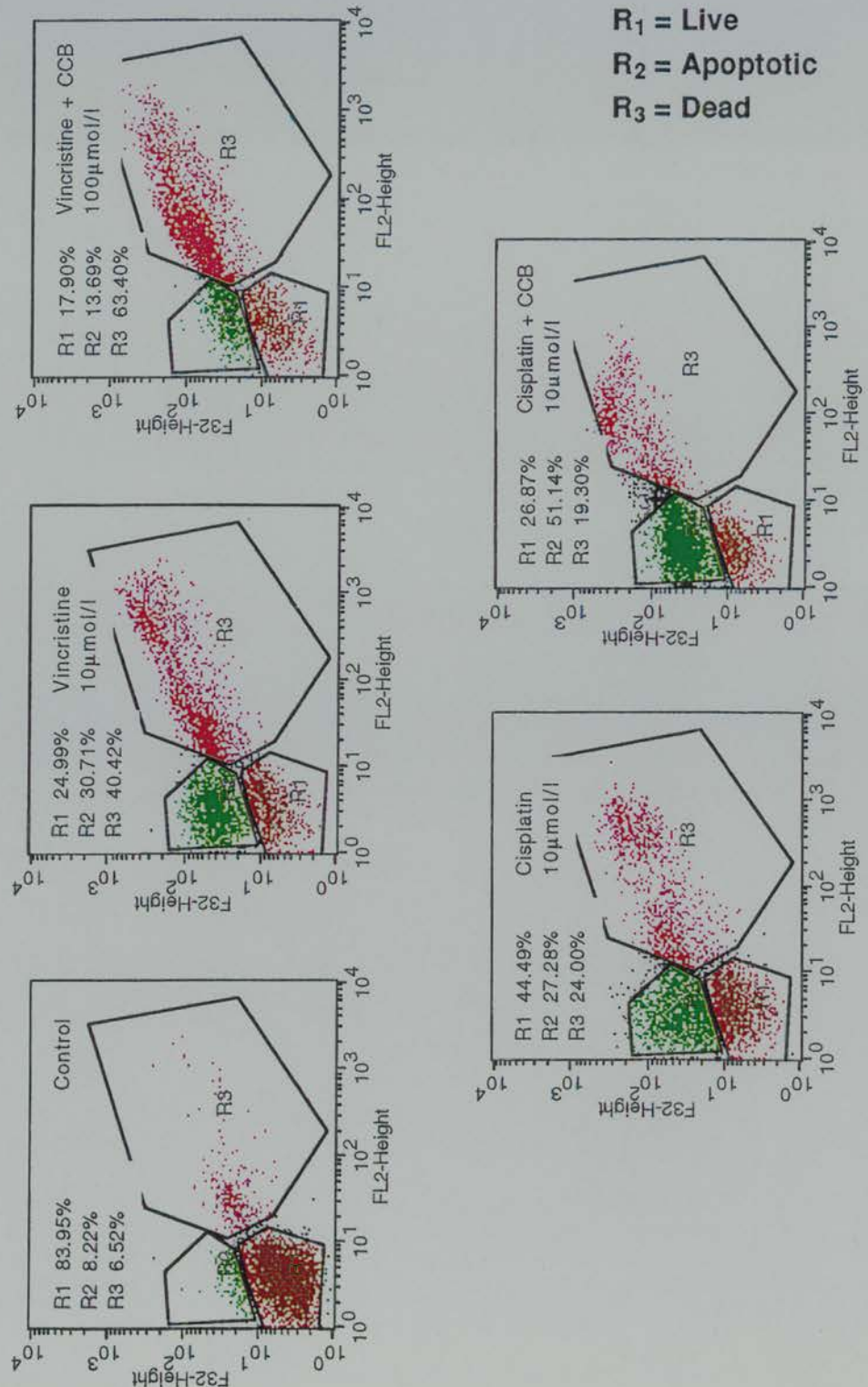


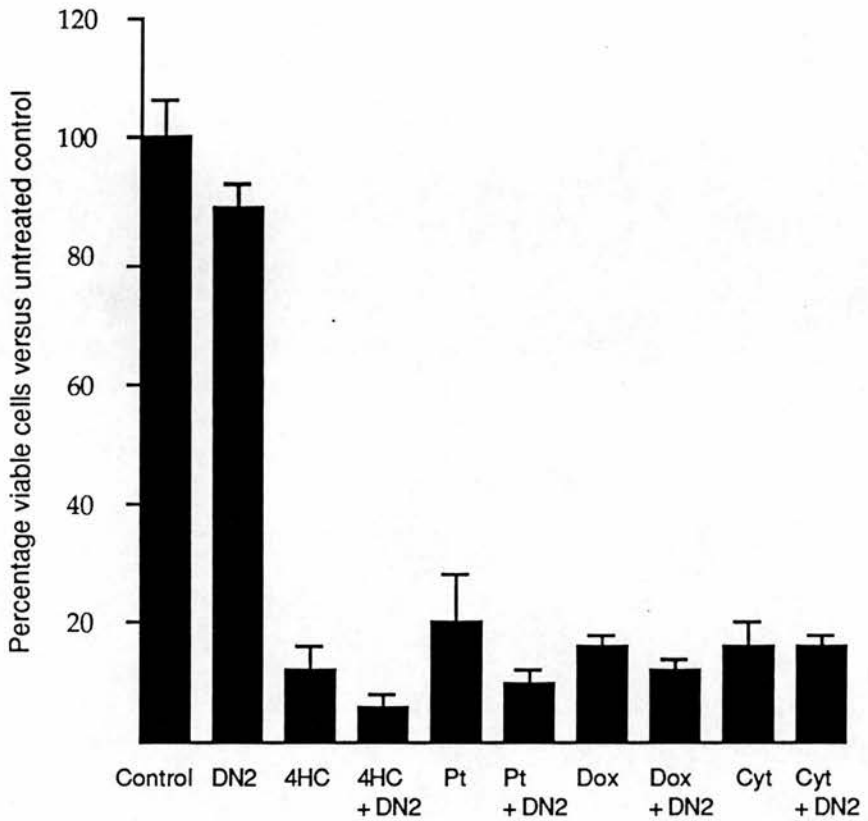
Figure 7: **Assessment of apoptosis in the $DOHH_2$ cell line 48 hours after treatment with Cisplatin or Vincristine alone or preceded by 72 hour incubation with Dantrolene (60μmol/L) and Nimodipine (1μmol/L) - calcium channel blockade**



(ii) 10 Day Experiment (see Figure 8)

Analysis was delayed until 10 days had elapsed rather than 5 in the previous experiment, because of evidence that the maximum effect of the calcium channel blockers may take 7 days to be seen. Here only a low concentration (dantrolene 6μmol/L, nimodipine 0.3μmol/L) was studied. A small antiproliferative effect (see graph) of the calcium channel blockers alone could be seen. Both 4HC and cisplatin - the two strongest inducers of apoptosis had evidence of enhancement by calcium channel blockade. The difference between cytotoxic drug versus cytotoxic drug and calcium channel blockade being significant ($p = 0.013$). Doxorubicin and cytarabine showed much weaker enhancement seen.

Figure 8: **10 Day analysis of calcium channel blockade versus chemotherapy alone. Chemotherapy given day 5-7**



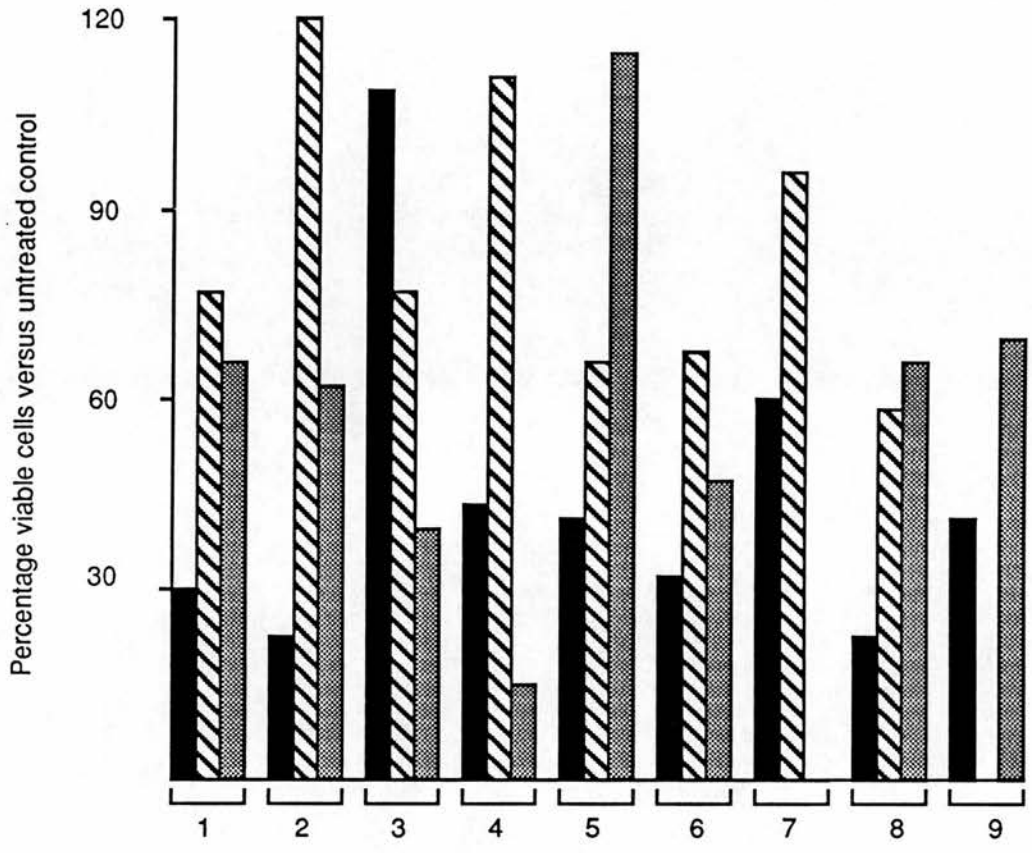
9.5 Drug sensitivity testing using the IL3/IL10 stromal cell system

9.5.1 *Cells analysed 48hrs after drug exposure - single agents - percentage versus untreated control - in the DOHH$_2$ cell line and two primary lymphoma cultures, L11 and L2*

L11 - Follicle centre lymphoma grade 3

L2 - Follicle centre lymphoma grade 1 (Figure 9)

Figure 9



Key

1 - Vincristine 10μmol/L
2 - Cytarabine 10μmol/L
3 - Dexamethasone 10μmol/L
4 - Methotrexate 10μmol/L
5 - Etoposide 10μmol/L
6 - Doxorubicin 0.1μmol/L
7 - Fludarabine 10μmol/L
8 - 4HC 10μmol/L
9 - Cisplatin 10μmol/L

Cell Line
L1105
L211

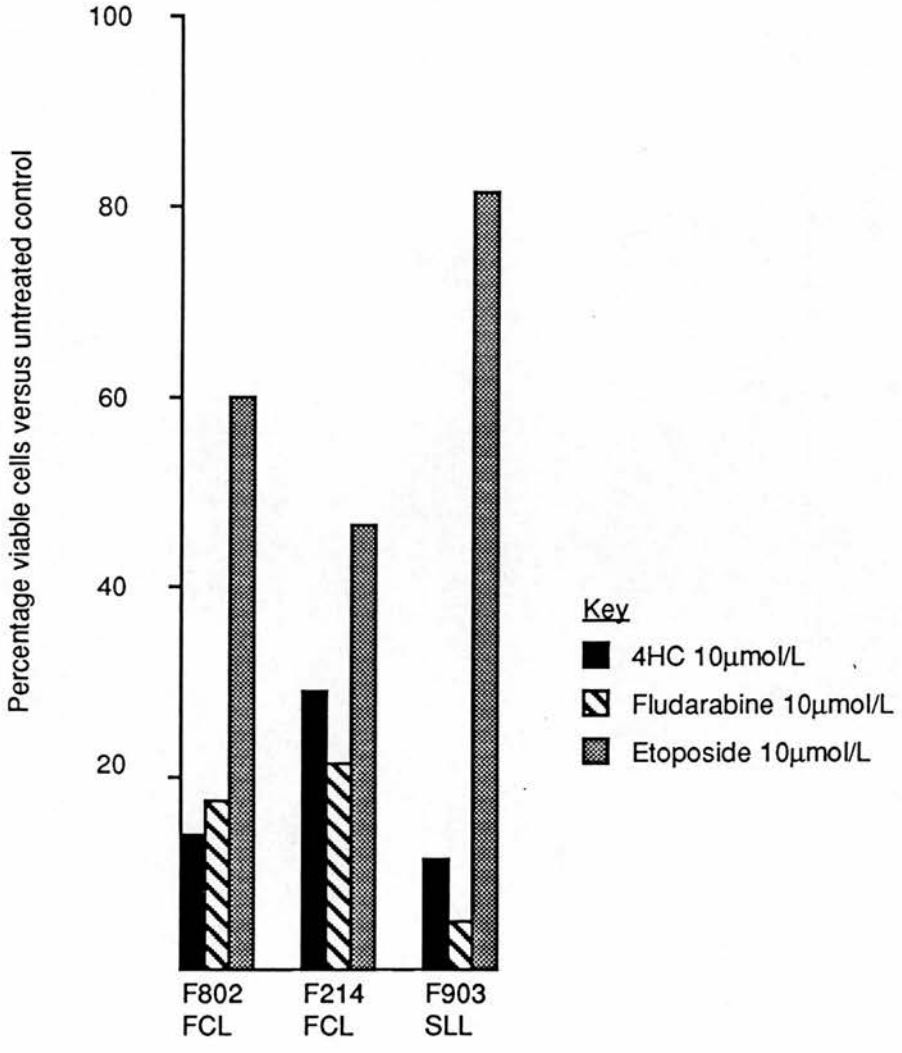
Overall the primary cultures seemed much less sensitive to cytotoxic drugs than the cell lines. 3 drugs which are schedule dependent - cytarabine, etoposide and fludarabine appeared relatively ineffective especially in L11. Dexamethasone was the

only drug which appeared consistently more effective in primary culture than in the cell line.

9.5.2 *Prolonged culture of fludarabine and etoposide (see Figure 10)*

To see if prolonged drug exposure enhanced the activity of these agents, both were left for 168hrs prior to analysis. The results were compared to 4HC.

Figure 10



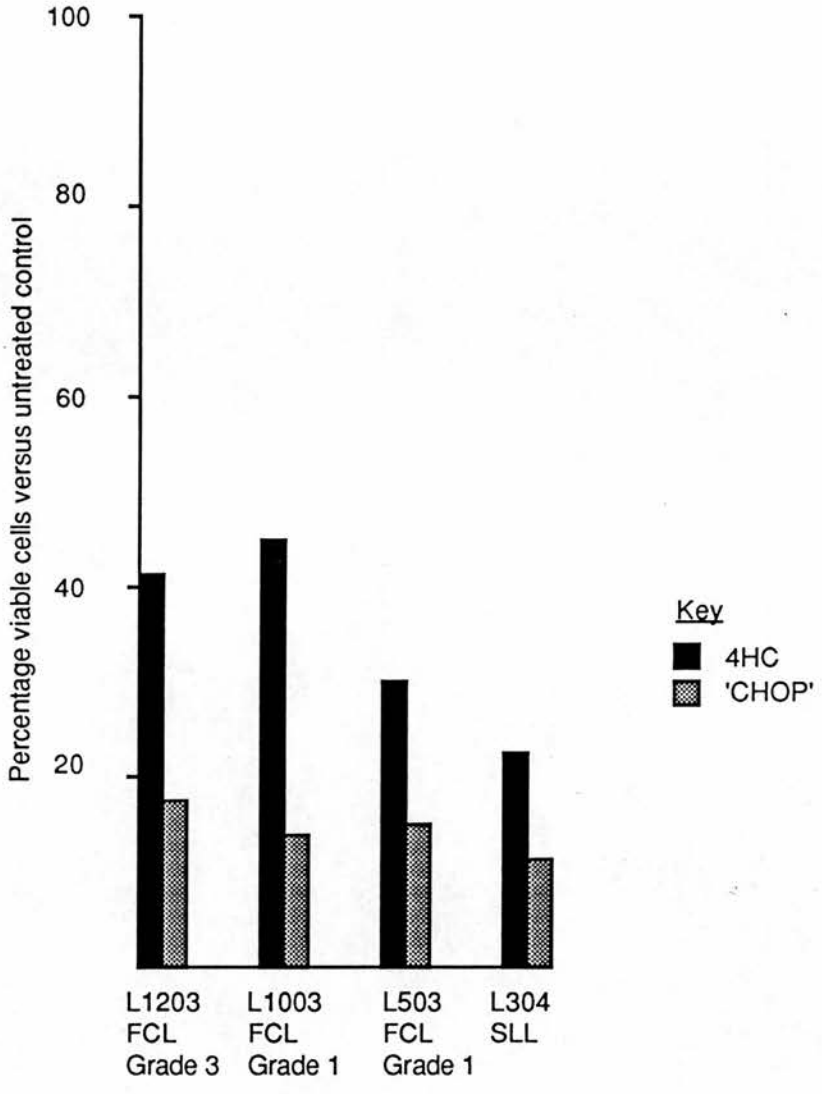
F8 - Follicle centre lymphoma, follicular grade 1
F2 - Follicle centre lymphoma, follicular grade 1
L 9 - B cell small lymphocytic lymphoma

Prolonged exposure lead to greater cell killing compared to the control in all cases. Interestingly the prolonged exposure showed fludarabine to be a more active agent than 4HC, whilst etoposide remained clearly weaker. Exposure timing may thus be critical for some drugs in this system.

9.5.3 *4HC versus 'CHOP' - see Figure 11*

This experiment utilised a 120hr period of drug exposure prior to cell counting. CHOP was represented by 4HC - 10μmol/L; vincristine 10μmol/L; doxorubicin 0.1μmol/L; dexamethasone 10μmol/L. 4 lymphomas were studied.

Figure 11



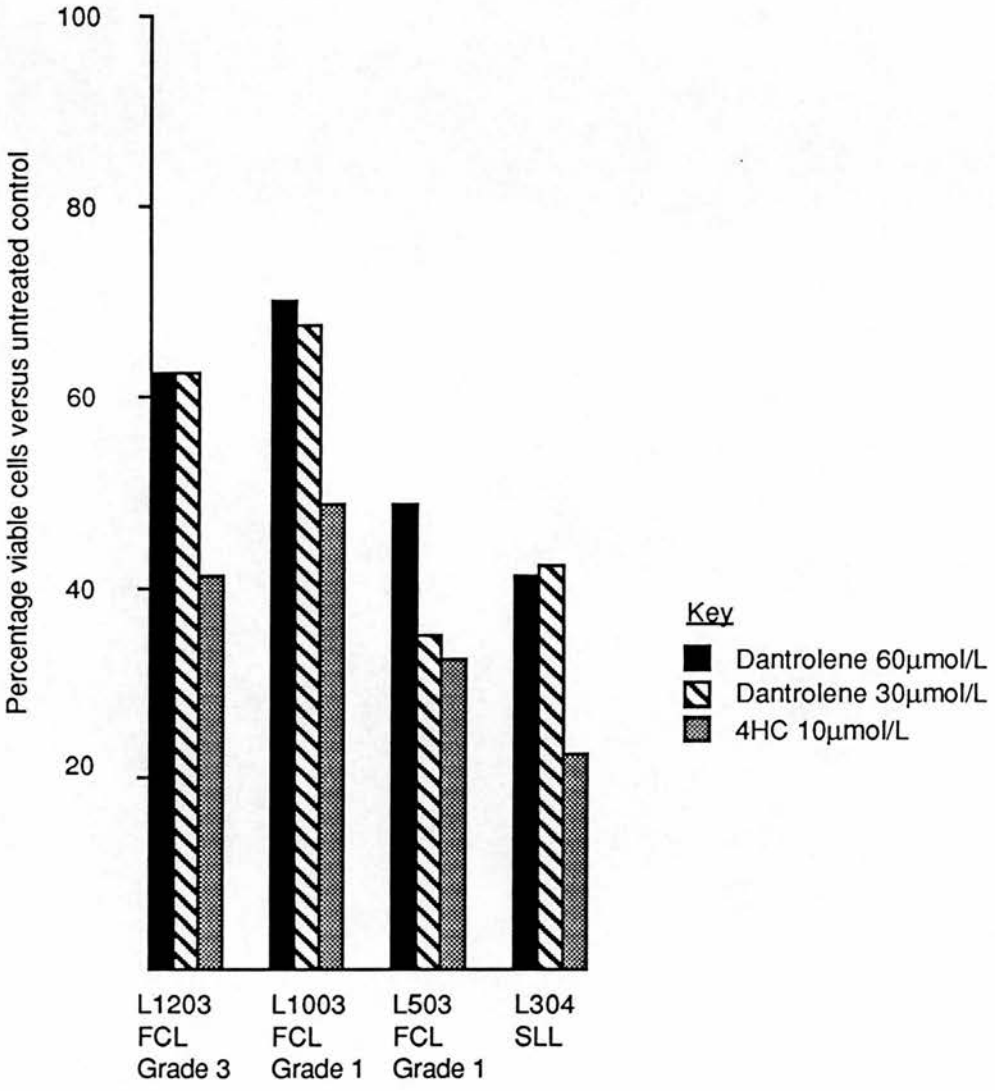
This result confirmed 'CHOP' to be superior to 4HC in the 5 day study described.

9.5.4 *Direct comparison of 4HC to dantrolene in low grade B cell lymphoma (see Figure 12)*

The results from the previous chapter with dantrolene were compared to 4HC. This confirmed that single agent dantrolene at

30-60μmol/L at a 5 day incubation was similar to 4HC in primary culture. There was little difference between 30 and 60μmol/L.

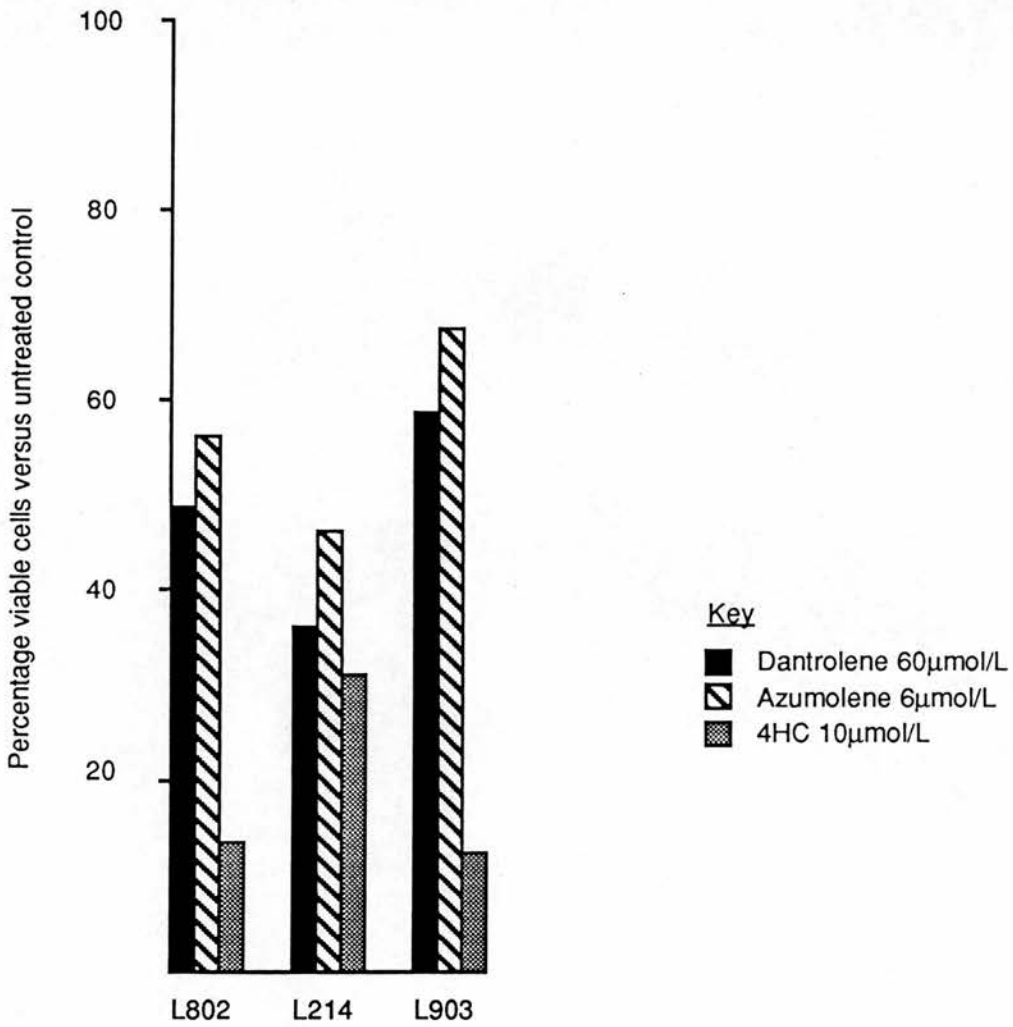
Figure 12



9.5.5 *Direct comparison of 4HC to dantrolene 60µmol/L and azumolene 6µmol/L in low grade B cell lymphoma (see Figure 13)*

The results of dantrolene and azumolene have already been shown but they are given with 4HC to allow a complete comparison to be made.

Figure 13

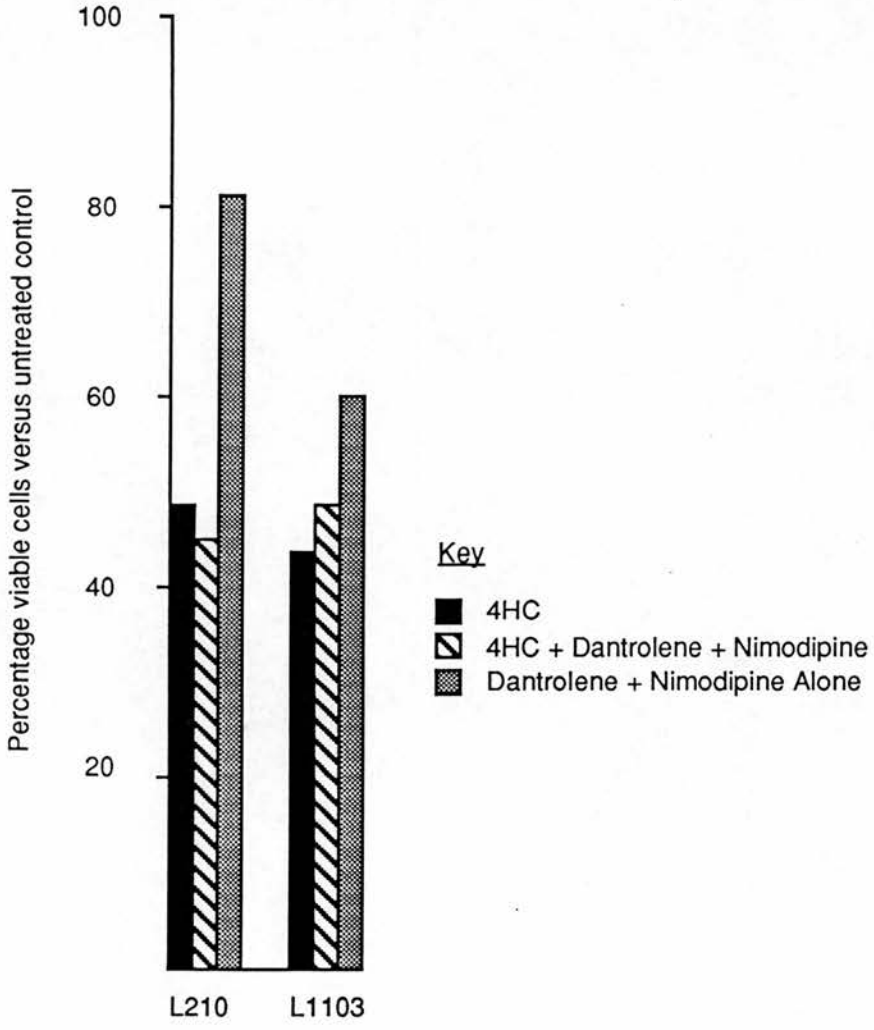


This result confirms azumolene and dantrolene to have significant activity, though less than that of 4HC in these studies.

9.5.6 *Combined calcium channel blockade with dantrolene, nimodipine and 4HC versus 4HC alone (see Figure 14)*

This experiment was carried out in two samples. Low concentrations of dantrolene and nimodipine were used (D - 6µmol/L; Nimodipine 0.3µmol/L) with 4HC - 10µmol/L for 48hrs.

Figure 14



No obvious difference could be seen, but the drugs were administered simultaneously - unlike the case in the $DOHH_2$ cell line.

9.5.7 *Direct comparison of cytotoxics versus azumolene in Hodgkin's lymphoma (see Figure 15)*

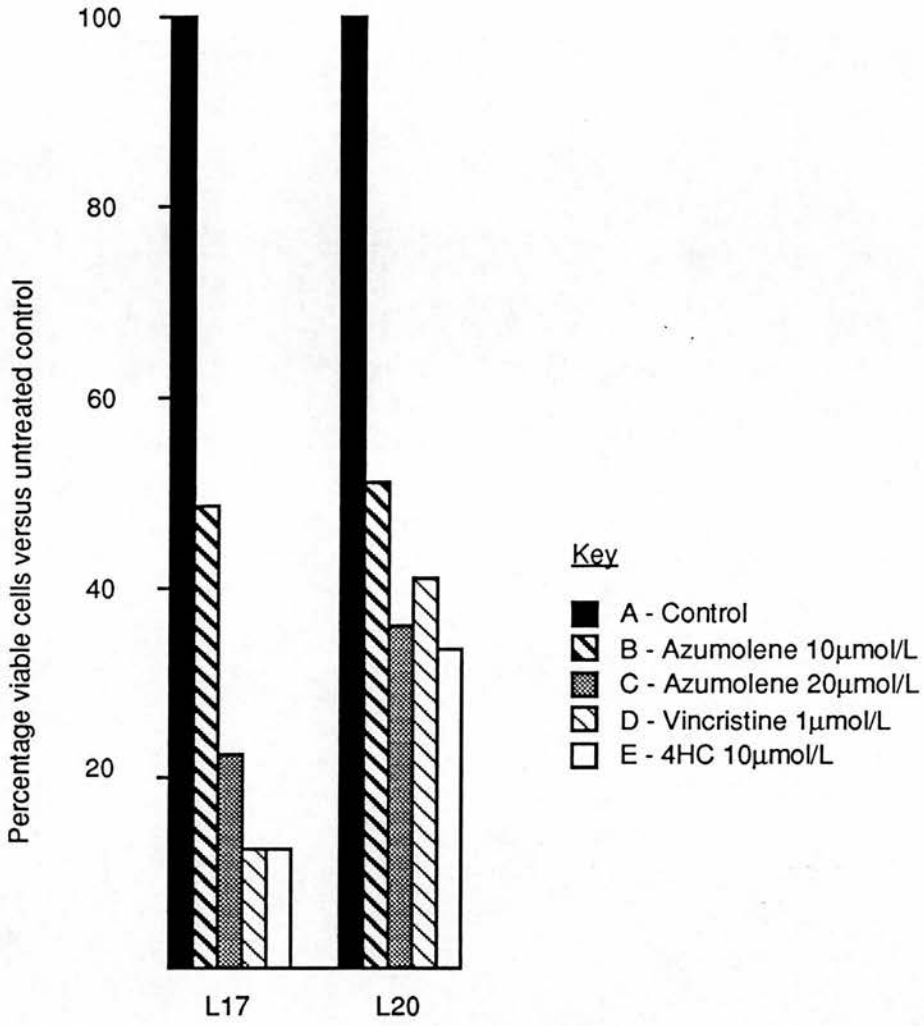
As CD15 and CD30 bearing cells make up only a small percentage of total, the treated groups and control had total cell counts and then FACS analysis to discover percentage of various subgroups. No T cell depletion was carried out. As CD30 is a B cell activated marker CD15 was used as the estimate of Hodgkin cells as this antigen was not the subject to upregulation via the cytokines/anti-CD40.

L1706 - Lymphocyte predominant HL.

L2005 - Lymphocyte predominant HL.

Experiment Design: The cells were cultured for 72hrs prior to drug administration, analysis was carried out 168hrs later (see below).

Figure 15



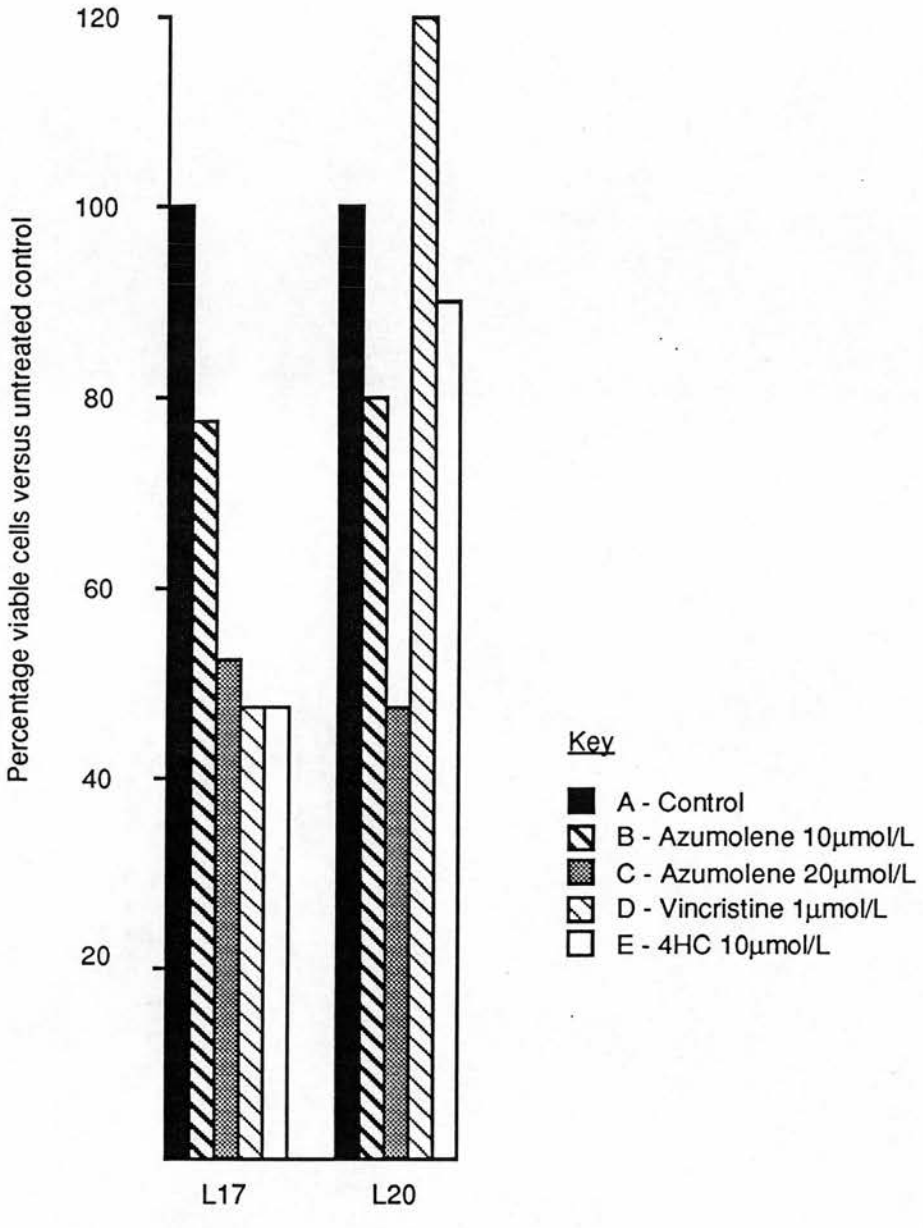
On the basis of the total cell count azumolene 20μmol/L appears equivalent to 4HC and vincristine. The phenotype of cultured cells is shown below (see Table 4). The number of CD3 bearing cells is much higher in the treated cells than in the untreated. The same is true of the number of CD15 bearing cells suggesting the number of B lymphocytes numbers reduced.

Table 4

FACS Analysis		L17			L20		
Ig	G_{1neg}	CD3	CD15	CD30	CD3	CD15	CD30
Control	0	27	12	8	40	10	9
Azumolene 10µmol/L	1	60	18	5	54	15	
Azumolene 20µmol/L	1	77	29	7	57	13	3
Vincristine 1µmol/L	1	82	40	22	69	26	6
4HC 10µmol/L	3	64	38	9	61	25	3

If the number of CD15+ve cells is calculated (%CD15 x total) cell count, the results are as follows (see Figure 16):

Figure 16: Total CD15 versus control



Using this analysis - all drugs appear less effective than when total cell counts are used. Azumolene is superior to cytotoxics in L20 and slightly less efficacious in L17. FACS analysis of treated cells is particuarly useful if the number of malignant cells within the culture is expected to be small and a fluorescently labelled antibody can identify them.

9.6 Discussion

The assessment of apoptosis using flow cytometry is not without problems. Perhaps the gold standard still remains microscopy[158]. When two methods morphology and DNA laddering are used - discrepancies can occur[159] and it has been suggested that more than one method should be used.

There is evidence that drugs with very different actions are able to induce apoptosis[120,158] and the cell type may be more important as to whether a cell undergo apoptosis or necrosis as opposed to the inducing drug. The evidence that the drugs which were the strongest inducers of apoptosis had this enhanced by calcium channel blockade with dantrolene and nimodipine is equivocal. Whilst the studies with vincristine and cisplatin in the $DOHH_2$ cell line suggested enhancement; in the 10 day experiment an enhanced effect was seen for cytarabine and doxorubicin (poor inducers of apoptosis) although this was weak; and may have represented independent cell killing. It seems very unlikely that the enhancement seen was due to P-glycoprotein inhibition. Neither the primary lymphomas cultured nor the $DOHH_2$ cell line appeared to possess functional P-glycoprotein pumps and there was no evidence from the peripheral blood lymphocytes (which did) that either dantrolene or nimodipine affected it.

The interpretation of the primary culture work suggests that the cytotoxics tested were less effective than in the $DOHH_2$ cell line. There was one major exception - dexamethasone which appeared to have no activity in the cell line whilst activity was demonstrated

in the primary culture. In one case methotrexate - a drug not known to have significant activity in low grade lymphoma appeared very effective (L211) this raises the question as to whether drug sensitivity testing in patients relapsing may reveal unexpected activity of certain drugs.

The timing of drug exposure appeared critical in certain cases especially with fludarabine. Whilst a 48hr exposure revealed the activity of 4HC - it failed to show activity of etoposide and fludarabine. On prolonging drug exposure to 168hrs the effect of fludarabine matched 4HC whilst although etoposide appeared to have some activity this remained inferior to the other two drugs. Experiments using a multidrug treatment - 4HC, doxorubicin, vincristine and dexamethasone - to represent CHOP and comparing this to 4HC alone showed it to be superior. This has not been borne out *in vivo* where there is little evidence of superiority of multidrug treatment over single agents - despite the fact that the initial response rate is higher[11].

Primary culture of solid tumours and haematological malignancies for 72hrs in RPMI base[160] has shown that different topoisomerase II inhibitors have markedly different activities with non-cross resistance being demonstrated - whether this translates into the *in vivo* situation is unclear. It has been shown that drug sensitivity testing is possible in HL although with only two samples studied it is unclear how consistent results will be. The small number of putative malignant cells has required that FACS analysis be

carried out, lest the fall in total number be due to a fall in benign lymphocytes

The testing of the ryanodine receptor antagonists in primary culture was described in the last chapter - the results being presented against single agent 4HC show the effect of these agents. Both dantrolene and azumolene appear less effective than 4HC although a higher concentration of azumolene needs to be tested and a method to allow pulsed administration as in the cell lines to see if this is more effective requires development. The limited data presented suggest that there may be little benefit in combining dantrolene and nimodipine with 4HC.

In conclusion based on initial cell line work to obtain appropriate concentrations of cytotoxic agents it has been possible to show that it is practical to evaluate drug sensitivity using the IL3/IL10 stromal cell system. In principle, a result would be available 10 days after the start of culture (3 days required to ensure the cells start to proliferate, 7 days continuous drug administration - necessary for fludarabine and etoposide). Such a time period to wait to decide on therapy is acceptable in many cases of low grade lymphoma. The correlation between the *in vitro* result and *in vivo* response would require prospective evaluation, however the fact that drugs which appear active in this system are known to be active *in vivo* (4HC, fludarabine) whilst drugs like cisplatin appear less active is reassuring.

CONCLUSION

The treatment of both low grade B cell and Hodgkin's lymphoma continues to be frustrating. In the low grade B cell lymphomas, the relapsing nature of the diseases in most patients even with the most intensive chemo-radio therapy regimens remains the main stumbling block. In Hodgkin's lymphoma - the curability of a majority of patients has lead many to try to find ways of maintaining the results whilst reducing toxicity; however, 20% fail to respond adequately to initial therapy and for them further conventional and even high dose chemotherapy still fail the majority of these patients. The lack of new drugs with which to make a difference in outcome as opposed to an alternative route to the current results has lead many to conclude that further advances in the treatment of these diseases is unlikely with conventional drugs and radiation and that a breakthrough will come from some form of immune or gene therapy - both seem unlikely.

The potential of the CD80 system to contribute to immune regulation has lead to renewed enthusiasm amongst those who favour tumour immunotherapy. The work presented in this thesis suggest that such an approach may be worth pursuing. Most low grade B cell lymphomas do not express high levels of CD80 and induction in culture certainly appears to make the cells more immunogenic as judged by a primary mixed lymphocyte reaction. The further development of such an approach depends on the ability to demonstrate a cytotoxic T cell response in autologous lymphocytes. It may be possible to enhance this using IL2 or other cytokines. Whether immunisation of patients with CD80 expressing lymphocytes will form the initial therapy or perhaps as is more likely therapy in those where substantial cytoreduction has been achieved with cytotoxics remains to be decided.

Perhaps the finding that azumolene and dantrolene have significant cytotoxic activity is of most practical relevance. How these drugs are killing the cells is unclear. Cell cycle interruption and a major induction of apoptosis are unlikely at least in the cell lines studied and their relatively slow action suggests a novel mechanism from conventional cytotoxics. The link to *bcl-2* expression - is tenuous although cells that produce high levels of *bcl-2* may be more sensitive. Azumolene in particular, in view of its higher therapeutic ratio than dantrolene appears to deserve further investigation in lymphoma management.

The use of primary lymphoma culture to predict drug sensitivity assumes that a large choice of agents exists. Whilst it may be appropriate in relapsing patients in whom first line drugs have failed, further data on reproducibility of results will be required before such a technique can become standard in planning therapy - in particular the ability to demonstrate significant activity of one agent as opposed to another is required.

The successful culture of Hodgkin's lymphoma comes at a time when there is renewed interest into the origin and pathogenesis of this disease, the low number of malignant cells within an affected node and the severity of cytokine deregulation in advanced disease and its relative sensitivity to cytotoxic agents all suggest that it may lend itself to different approaches in therapy. The search for infective triggers is likely to increase and it may be that as in some mucosa associated lymphoid tumours early treatment with anti-infective agents may reduce the requirement for cytotoxic chemotherapy.

To conclude - the demonstration of a relatively flexible technique to store and then culture lymphoma tissue from patients who have low grade B cell or Hodgkin's lymphoma and the ability to use this to investigate new approaches to therapy has been demonstrated. It is increasingly likely that additional modalities of treatment will join chemo and radiotherapy in the management of these diseases although their replacement by new techniques seems on current evidence unlikely.

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Leukemia (1995) 9, 1349–1352

Induction of CD80 expression in low-grade B cell lymphoma – a potential immunotherapeutic target

J Shamash[1], DC Davies[2], A Salam[1], AZS Rohatiner[1], BD Young[1] and TA Lister[1]

[1]*ICRF Department of Medical Oncology, St Bartholomew's Hospital; and* [2]*ICRF FACS Laboratory, London, UK*

The CD80 antigen (B7) is expressed on activated B lymphocytes. It is thought to be important in eliciting a T cell response via its ligands CD28 and CTLA-4 when antigen is presented in the presence of the MHC-1 peptide. Low-grade B cell lymphomas analysed by flow cytometry express CD80 very poorly. However, when grown *in vitro* using the IL-4/anti-CD40 stromal cell culture system, following depletion of T and IgD-bearing cells, a monoclonal B cell expansion occurs. Cells harvested at days 10–13 express the antigen strongly, regardless of the histological subtype of lymphoma. Further investigation of CD80-mediated immune functions may be possible using this system as a basis for testing immunotherapy.
Keywords: CD80 expression; low-grade lymphoma

Introduction

CD80[1] (B7) is an antigen of the immunoglobulin super family which was originally described as being present on activated B lymphocytes and not circulating B cell malignancies.[2] It has also been found on HTLV-1 infected T cells[3] and cells of the monocyte–macrophage series.[4] It is a cell membrane protein with a short, highly charged cytoplasmic tail;[2] the extracellular region resembles that of immunoglobulins. Recently, it has been suggested that there might be three members of the B7 family (B7-1, B7-2 and B7-3)[5] which could act as ligands to CTLA-4[6] and CD28,[7] both T cell antigens that bind B7.[6] CTLA-4 appears to be homologous to CD28 and has been cloned to the same chromosomal region. Following T–B cell interaction over a period of 72 h the expression of different members of the B-7 family may be induced.[5] The studies described below only examined CD80 (B7-1) expression.

In addition to the cell–cell interaction, the generation of T lymphocyte immune responses requires soluble mediators (cytokines) and accessory surface antigens. CD80 falls into the latter group.[9] The specificity of the T–B cell interaction depends on the T cell receptor (TCR)/CD3 complex and the antigen associated with class II MHC molecules on B cells; without accessory molecule function full activation of the T cells is not possible.[8]

Recent murine studies exploring the potential contribution of accessory surface antigens are provocative: the introduction of CD80 onto the cell membranes of murine melanomas is sufficient to elicit a T cell response which can stop tumour growth *in vivo*.[10] Moreover, pre-vaccinating mice with such cells prevents non-CD80 expressing melanoma cells of the same type from growing when the same mice are re-challenged 2 weeks later. *In vitro* expansion of low-grade lymphoma, followed by induction of CD80 expression could possibly be used as a source of cells which could be reinfused as a form of 'immunotherapy'.

Initial reports on CD80 expression[2] suggested that follicular lymphomas express CD80.[2,9] It has been reported that IL-4 can upregulate CD80 following the stimulation of B lymphocytes with anti-IgM or *S. aureus* Cowan (SAC).[1] Cross-linking of the B cell antigen CD40 is an alternative way of activating B lymphocytes. These will undergo long-term proliferation[11] when incubated with irradiated fibroblasts (Ltk cells) transfected with the human monocyte Fc receptor in the presence of IL-4. A similar response has been observed in follicular lymphoma cells.[12]

A study was therefore undertaken to confirm whether the low-grade B cell lymphomas express the CD80 antigen and whether or not they might be cultured for long enough to allow transfection of the gene for CD80. The results below suggest that transfection may not be necessary.

Materials and methods

Preparation of the lymphoma cell suspension

Twelve fresh specimens of confirmed low-grade lymphoma (eight follicular, two lymphoplasmacytoid, one diffuse centrocytic/centroblastic and one small lymphocytic, according to the Kiel classification) were dissected and cell suspensions prepared in RPMI 2%, fetal calf serum 5% (Gibco, Paisley, UK), gentamicin 40 μg/ml (Roussel Laboratories, Uxbridge, UK) and Hepes 25 mm (Gibco). Centrifugation on Ficoll–Hypaque (Lymphoprep; Nycomed, Oslo, Norway) was carried out to isolate the mononuclear cell fraction. This was fluorescence immunophenotyped using anti-BB-1 (B7) at a dilution of 1 : 160/100 μl/10^6 cells (Becton Dickinson, Oxford, UK). Mouse IgG1 (Becton Dickinson) was used as a negative control. Phenotyping was performed on a FACScan. Fluorescein isothiocyanate (FITC) staining was detected using a 530/30 nm band pass filter and phycoerythrin (PE) staining with a 585/42 nm band pass filter. Non-lymphoid cells were gated out using forward and side scatter; 2000 cells were surveyed in all FACS analyses, the remaining cells being frozen as a suspension in RPMI 2% with 20% fetal calf serum (Gibco) and 10% DMSO (Fluka, Buchs, Switzerland) at a concentration of 2×10^7 cells/ml.

Thawing of cell suspensions

The cells were thawed and then resuspended in Dulbecco's without phenol red (Gibco) with 1% bovine serum albumin (Sigma, Poole, UK) and human immunoglobulin 0.5 g/l (Sandoz Pharmaceuticals, Berne, Switzerland). They were subsequently immunophenotyped using the following antibodies: IgG1 FITC negative control (mouse) (Serotec, High Wycombe, UK), anti-CD3 FITC (Dako), anti-CD14 FITC (Dako), anti-IgD polyclonal $F(ab)_2$ FITC (Dako). Two-colour fluorescence was carried out using anti-kappa polyclonal $F(ab)_2$ FITC (Dako), anti-lambda polyclonal $F(ab)_2$ PE (Dako).

Correspondence: J Shamash, Department of Medical Oncology, St Bartholomew's Hospital, West Smithfield, London, EC1A 7BE, UK
Received 24 October 1994; revised 24 April 1995; accepted 1 May 1995

Depletion of T and IgD bearing cells

2×10^7 lymphoma cells were incubated with anti-IgD (Dako) at a concentration of 100 μl/1 ml/10^7 cells, anti-CD8 (Dako), 50 μl/1 ml/10^7 cells and anti-CD4, 250 μl/1 ml/10^7 cells. Depletion of T cells and IgD-bearing cells was carried out after 30-min incubation, with Dyna beads (R) (Dynal International, Oslo, Norway) coated with anti-mouse Ig 1 ml/10^7 cells on a rotating mixer at 4° C for 1 h followed by magnetic separation. The remaining cells were immunophenotyped using the following antibodies: IgG negative control, anti-CD3 FITC, anti-IgD $(Fab)_2$ FITC, anti-CD19 FITC, (all Dako). B7-24 (anti B7, IgG2a kappa) (kindly supplied by Innogentics, Zwijnaarde, Belgium),[13] with a second layer anti-IgG2a FITC (kindly supplied by Dako), anti-CD11a FITC, anti-CD54 FITC, anti-CD58 FITC (kindly supplied by Dako), anti-CD54 FITC, anti-CD58 FITC (all Serotec), IgG2a FITC control (Dako), anti-BCL-2 FITC (Dako) and anti-HLA-DR FITC (Dako).

Comparison of B7-24 and BBI

BBI and B7-24 (Becton Dickinson) were compared using a Raji cell line (known to express CD80) and were found to be equivalent. B7-24 was used to assess CD80 expression in resuspended cells because its IgG2a class allowed it to be used in the presence of anti-CD40 (IgG1) the second layer anti-IgG2 being specific to the subgroup.

Culture system

The cells were grown using the anti-CD40/IL-4 stromal cell system.[11] Mouse fibroblasts Ltk cells transfected with the CDw 32 Fc receptor obtained from the American Tissue Culture Collection (with the permission of Dr KW Moore), were grown to confluence in 24-well culture plates and then irradiated to 75 Gy. The lymphoma cells were suspended at a concentration of 5×10^5/ml, with 2 ml per well, in a growth medium consisting of Iscove's modified Dulbecco's medium without FCS (Gibco), with 50 μg/ml human holotransferrin, 0.5% bovine serum albumin, 5 μg/ml/ bovine insulin, long chain fatty acids (*cis*-9 octadecenoic, all *cis* 9,12 octadecadienoic, and hexadecanoic acid all 1 μg/ml (all from Sigma), 2% v/v fetal calf serum, penicillin and streptomycin (Gibco) and gentamicin 40 μg/ml (Roussel) anti-CD40 1 μg/ml (Serotec) and hIL-4 46 ng/ml (kindly provided by Schering Plough Research, Bloomfield, NJ, USA). Cells were grown on the fibroblast monolayer for 10–13 days before pippetting out and re-phenotyping with the same antibodies.

Results

Initial phenotyping of samples

Fresh: Immunophenotyping of fresh samples revealed poor expression of CD80. The median proportion of cells expressing CD80 compared to control was 9% (standard deviation (s.d.) 24, range 2–89%). There was no evidence of a discrete CD80-positive population of the cells.

Frozen: Phenotyping of the same lymphoma cells following thawing using the antibody B7-24 revealed a median expression of 8% (s.d. 16) range 1–59%.

Cell viability

Viability of resuspended cells was assessed using 0.2% trypan blue exclusion, a median viability of 95% (s.d. 3) was seen in the 12 samples studied.

Confirmation of T and IgD bearing cell depletion and clonality

The use of paramagnetic beads reduced the proportion of T cells from a mean of 34% (s.d. 20) to 8% (s.d. 7) and IgD-bearing cells from 22% (s.d. 17) to 6% (s.d. 7). The cells showed light chain restriction using two-colour staining.

Bcl-2 and adhesion molecule expression

The cells showed low but significant expression of Bcl-2. Median expression of CD11a, CD54 and CD58 was 34% (s.d. 19), 50% (s.d. 17) and 84% (s.d. 17) respectively.

Analysis of cells harvested after 10–13 days

Increased expression of CD80 was shown by a shift in medial channel fluorescence, overall fluorescence increasing from 16.08 (s.d. 22) to 62.7 (s.d. 17) (individual results with corresponding histology are shown in Table 1 and Figure 1, two individual histograms are shown in Figure 2).

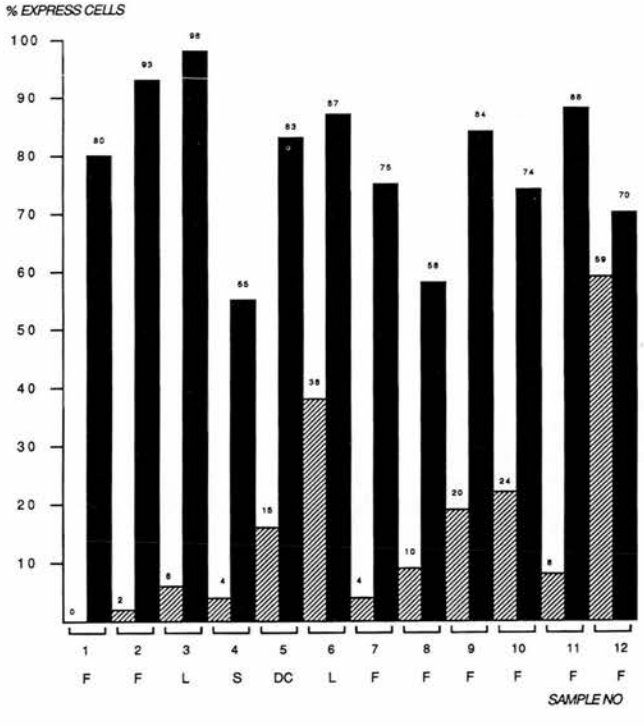

Figure 1 Expression of CD80 (B7-24) compared to IgG2a control isolated low-grade lymphoma B cells before and 10–13 days after growth in the IL-4/anti-CD40 stromal cell culture system

Table 1 Characteristics of cultured cells at days 10–13

Sample	*Histology*	*CD3*	*IgD*	*CD19*	*Light chain*	*CD80*	*CD11A*	*CD54*	*CD58*
1	F	3	40	53	61κ	80	81	NA	99
2	F	9	2	68	95λ	93	87	96	99
3	L	12	49	82	63κ	98	53	98	100
4	S	4	3	51	99λ	55	30	96	97
5	DC	6	33	58	69λ	83	64	90	98
6	L	14	2	47	98λ	87	49	66	95
7	F	2	41	57	95κ	75	31	93	97
8	F	17	10	25	76κ	58	39	68	94
9	F	7	18	50	78κ	84	35	57	100
10	F	19	65	59	61κ	74	89	98	99
11	F	5	18	71	75κ	88	61	91	99
12	F	9	50	65	NA	70	89	NA	99

F, follicular lymphoma; S, small lymphocytic; L, lymphoplasmacytoid; DC, diffuse centrocytic/centroblastic; NA, not available

Details of individual lymphomas are shown in Table 2. During culture a B cell proliferation occurred without evidence of T cell overgrowth. The cells continued to show light chain restriction on FACS analysis but there was a shift in phenotype with an increase in CD19+ve cells and an upregulation in the adhesion molecules studied. Expression of CD11a, CD34 and CD58 increased to 58% s.d. 22, 92% s.d. 14 and 99% s.d. 2, respectively.

In four lymphomas studied for HLA-DR expression this was high at the end of the culture period.

Discussion

The stromal cell culture system offers a useful way of investigating various aspects of the growth characteristics of low-grade lymphoma and provides the potential to enhance expression of certain antigens. Previously, it had only been used to culture follicular lymphomas.[13] It can now be confirmed that it supports the growth of other low-grade B cell malignancies. The ability to freeze cells and then resuspend and grow them offers further flexibility.

In the past, a drawback has been the apparent change in phenotype with a loss of mature cell antigens and the development of blast-like morphology;[13] this has been seen in all lymphomas studied here. However, the alteration and inter-lymphoma variation of CD19 and IgD-bearing cells does not appear to affect CD80 expression or the expression of the adhesion molecules studied. Thus, IL-4 can induce CD80 expression in benign[11,15] and malignant B cells; cross-linking of CD40 alone[5] can also induce expression of it in benign cells.

To achieve sustained proliferation of low-grade lymphoma *in vitro* requires both IL-4 and CD40. There appears to be a positive feed-back loop in the inter-regulation of CD80, IL-4 and CD40, in that ligation of CD80 with CD28 is itself able to induce IL-4 secretion from B cells in benign lymphocytes.[14]

The current conventional treatment of low-grade lymphoma is with relatively innocuous chemotherapy usually resulting in the induction of a partial remission; more intensive therapy gives a higher frequency of clinical remission but recurrence or progression is usual. The concept of using a form of immunotherapy to convert a clinical remission into cure is attractive. Re-infusion of a patient's own lymphoma cells following *in*

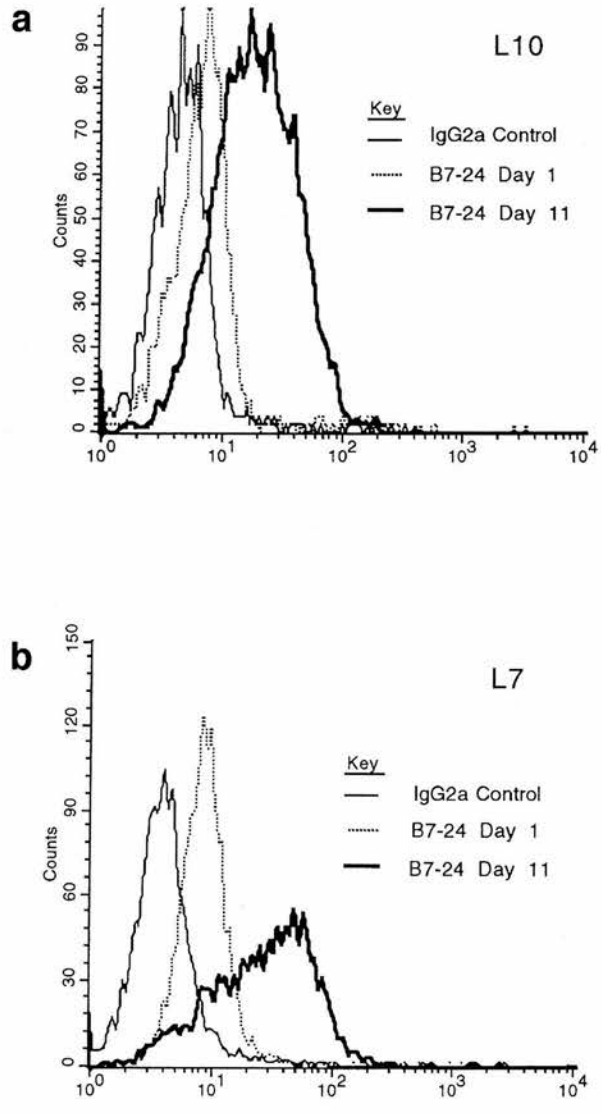

Figure 2 Two examples of low-grade lymphoma showing expression of CD80 (using B7-24) pre and post culture as analysed by FACS

Table 2 Pre-culture B cell characteristics following IgD and CD3 bearing cell depletion

Sample	*Histology*	*CD3*	*IgD*	*CD19*	*Light chain*	*CD80*	*CD11A*	*CD54*	*CD58*
1	F	13	13	62	90κ	0	36	68	87
2	F	7	0	27	97λ	2	33	64	62
3	L	2	2	38	65κ	6	53	37	42
4	S	7	1	84	99λ	4	32	20	86
5	DC	5	2	35	92λ	15	61	49	72
6	L	14	1	71	99λ	38	26	49	72
7	F	4	6	45	96κ	4	22	54	96
8	F	6	1	13	95κ	10	12	44	43
9	F	3	8	70	97κ	20	45	51	84
10	F	20	3	12	83κ	24	23	23	65
11	F	2	16	70	97κ	8	79	70	77
12	F	16	24	30	74κ	59	64	75	NA

Abbreviations as for Table 1

vitro expansion and induction of CD80 expression might even be considered provided that the patient's T cell competence was confirmed.

Acknowledgements

We thank Professor P Beverley at the Middlesex Hospital, London for his advice and assistance in preparing this work, the Department of Radiotherapy at St Bartholomew's Hospital, London for arranging the irradiation of the fibroblast monolayer and Dr Andrew Norton in the Department of Histopathology, St Bartholomew's Hospital, London for reviewing the histology and Stephanie Thomas for typing the manuscript.

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